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THE EFFECTS OF ADRENALECTOMY ON COBALT POLYCYTHEMIA IN THE RAT

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ABSTRACT

Sixty-nine male Wistar (Purdue) rats were studied in three separate experiments. Daily intraperitoneal injections of cobalt chloride (2.5 mg. of elemental cobalt per kg. body weight) were given for 30 to 65 days to normal, adrenalectomized and sham-operated rats.

Adrenalectomized rats treated with cobalt when compared with normal controls showed a decrease in blood values 20 days after adrenalectomy similar to adrenalectomized controls. Normal and sham-operated rats treated with cobalt showed a significant ($P < .01$) elevation in the erythrocyte count, hematocrit and hemoglobin after 20 days. During the period 30–65 days after the operation the adrenalectomized rats treated with cobalt did show a significant elevation of blood values which is explained on the basis of the development of accessory adrenal cortical tissue. Other significant increases were: ratio of adrenal to body weight in normal animals given cobalt; red cell volumes in all cobalt treated animals at 60 days; MCV and MCH values at 20 days in all adrenalectomized rats with or without treatment with cobalt.

WALTNER and Waltner (1, 2) found that cobalt induced a polycythemia in the rat and other animals. Several investigations (3, 4, 5) of bone marrow *in vitro* have not elucidated the mechanism of cobalt polycythemia. Hypophysectomy, which produces anemia in rats, did not prevent cobalt polycythemia (6, 7). However, involvement of the adrenal gland is suggested by several factors which indicate that the adrenocortical hormones play a regulatory role in hemopoiesis. In Cushing's Disease a polycythemia is associated with an increased secretion of the adrenal cortex (8). A pituitary extract free of all known pituitary hormones except ACTH produced a polycythemia when given to normal rats (9). Bone marrows from rats given prolonged injections of adrenocortical extract showed an increase in the "stem" cells of the erythrocytic series (10). This

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is strikingly similar to the intense proliferation of the erythrogenic cells in the bone marrow of rats treated with cobalt (11). Hydrocortisone and cortisone, alone or in combination with desoxycorticosterone, stimulate erythropoiesis in the adrenalectomized rat (12, 13). The experiments presented here compare the changes induced by cobalt in the blood of the adrenalectomized rat with the changes seen in normal rats treated with cobalt.

MATERIALS AND METHODS

Male albino rats of the Wistar (Purdue) strain fed Purina Laboratory Chow were used. The adrenalectomized rats were maintained on 0.9% NaCl given as drinking fluid. Blood was obtained for erythrocyte, hemoglobin and hematocrit determinations by anesthetizing the rat with ether and clipping the tail. Erythrocyte counts were done using U. S. certified blood pipettes and the improved Neubauer counting chamber. Hematocrit determinations were made with Van Allen hematocrit tubes (1.6% aqueous sodium oxalate being used as a diluent) which were spun for 30 minutes at 3000 r.p.m. with a radius of 17 cm. Hemoglobin determinations were made on the Klett-Summerson Photoelectric Colorimeter using the acid hematin method (14). Rats were weighed at the time of the hematologic studies.

The erythrocyte blood volume was determined with P^{32} tagged erythrocytes (15). They were injected into the vein on the inner aspect of the thigh and blood samples removed via heart puncture for counting after 10 minutes. Counts were made on aluminum planchets and good adherence and surface geometry were achieved by the use of gum.

Cobalt chloride[2] was given by intraperitoneal injection daily (6 days per week) at a dosage of 2.5 mg. of elemental (10 mg. $CoCl_2 \cdot 6H_2O$) cobalt per kg. of body weight. Bilateral adrenalectomy was performed by making a short transverse incision (1–2 cm.) through the skin and lumbar muscles just below the last rib on each side. In order to insure against leaving behind any closely adhering accessory adrenal tissue, the fat surrounding the gland to a distance of ½ to 1 cm. was removed on each side. One stitch on each side was sufficient to close the incision. Each adrenalectomized animal was given 10,000 units of Procaine Penicillin intramuscularly at the time of the operation and on the following day. At the end of the study, the adrenals were removed from the cobalt-treated intact animals and normal controls; frozen sections were made and stained with Sudan IV and hematoxylin. Any accessory adrenal tissue found in the adrenalectomized groups was weighed, fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Blood smears were made from animals in the adrenalectomized groups, stained with Giemsa and observed microscopically for the presence of Bartonella muris. None of the animals tested showed signs of Bartonella infection.

Experiment I. Hematologic studies were made in 3 control, 6 cobalt-treated, 6 adrenalectomized and 5 adrenalectomized cobalt-treated animals (except for 1 animal dying after 10 days) for a period of 65 days after operation. Daily cobalt injections were started 3–5 days after adrenalectomy in the adrenalectomized-cobalt-treated group. Blood values were determined on the 10th, 20th, 40th and 65th day. The animals were then sacrificed and the adrenals weighed.

Experiment II. Hematologic studies were made in 8 control, 10 cobalt-treated, 8 adrenalectomized and 8 adrenalectomized cobalt-treated animals at 10-day intervals for a period of 60 days after operation. An initial 10-day control observation period was carried out in which blood values were determined at 10, 5 and 0 days before treatment.

[2] Analytical grade $CoCl_2 \cdot H_2O$ with the maximum limits of impurity for lead of 0.005%.

TABLE 1. RESULTS OF COBALT TREATMENT ON THE BLOOD PICTURE OF THE ADRENALECTOMIZED AND NORMAL MALE ADULT RAT (EXPERIMENT I)

Day of Treatment	Non adrenalectomized		Adrenalectomized	
	Controls	Cobalt-treated*	Controls	Cobalt-treated*
	Erythrocytes in millions per cu. mm.			
0	8.6± .78	8.4± .60	8.6±1.1	9.0± .51
10	8.3± .36	10.0± .95	8.0±2.2	8.1±1.2
20	8.8± .26	10.5± .85	8.4±1.3	7.7± .94
30	9.7± .59	10.7±1.9	10.1± .8	10.1± .70
40	9.3± .88	11.9±1.5	9.3±1.2	11.7± .26
65	8.0±1.2	14.8±1.4	9.2±1.4	12.9±1.4
	Hemoglobin in gm. per 100 ml.			
0	15.3±1.3	14.2± .78	14.4±1.1	14.0± .6
10	14.9±1.7	16.0± .83	12.7±1.3	14.4± .99
20	14.7± .24	17.0± .67	13.9± .57	14.4± .83
30	15.8± .33	18.9± .63	13.5±1.3	16.1± .64
40	15.9±1.3	20.0± .43	14.7± .98	17.2± .84
65	14.6± .69	19.8± .71	13.7±1.7	18.8± .46
	Hematocrit in per cent			
0	42.7±2.1	41.0±3.7	39.2±4.1	38.0±4.2
10	42.3± .57	46.2±1.6	35.8±1.3	39.0±2.7
20	43.7±3.1	51.0±2.3	40.3±3.9	44.3±4.3
30	46.0±2.6	56.2±3.6	42.2±4.8	46.0±6.0
40	47.0±4.1	59.3±5.7	40.3±2.8	55.5±3.8
65	45.0±1.0	68.5±3.0	41.4±4.5	67.6±2.9

* Cobalt dosage was 2.5 mg. of elemental cobalt per kg. daily i.p.
± = Standard Deviation.

Daily cobalt injections were started 24 hours after the operation in the adrenalectomized cobalt group. Total red cell volumes were determined with P^{32} tagged erythrocytes on the 60th day of treatment.

Experiment III. Changes in hematocrit were observed in 5 control, 5 cobalt-treated (2.5 mg. elemental cobalt per kg.) and 5 sham-operated cobalt-treated animals initially and at 10-day intervals for 30 days. The 5 control and 2 animals from each of the cobalt and cobalt-sham-operated groups were sacrificed at 30 days and the adrenals weighed. The additional animals in the two remaining groups received no further cobalt injections but hematocrit determinations were made at 40, 50 and 60 days to determine the rate at which the blood values returned to normal.

RESULTS

Experiment I

A. *Peripheral Blood.* From the 10th to the 65th day of treatment the erythrocyte count, hemoglobin and hematocrit were significantly ($P = .01$ except .08 at 40 days) elevated in the cobalt treated intact animals (Table 1). The adrenalectomized and adrenalectomized-cobalt groups became mildly anemic at 10 and 20 days. However, after 30 days the adrenalectomized group approached the controls in erythrocyte counts but remained below in hematocrit and hemoglobin. The erythrocytes, hemoglobin and hematocrit values of the adrenalectomized, cobalt-treated group were well above those for both normal and adrenalectomized controls after 30 days.

B. *Adrenal Weights and Histology.* The cobalt-treated non-adrenalecto-

TABLE 2. THE EFFECTS OF COBALT CHLORIDE INJECTIONS ON THE ADRENAL WEIGHTS IN ADULT RATS

Experiment No.	Day of Treatment	Controls		Cobalt treated*		P values
		No. of animals	Adrenal weight mg./100 gm.	No. of animals	Adrenal weight, mg./100 gm.	
I	65	3	9.8±2.6	6	23.8±2.2	.01
II	60	8	14.3±1.7	9	22.8±3.1	.01

* = Dosage was 2.5 mg. elemental cobalt per kg. daily (i.p.).
± = Standard Deviation.
P = "t" test of significance—controls vs. cobalt group.

mized rats showed a moderate to marked depletion of lipid in the cortex and a significantly ($P < .01$) greater adrenal to body weight ratio than controls (Table 2).

Accessory cortical tissue in rats was in many instances difficult to find. Macroscopic accessory adrenals, which were confirmed by microscopic examination, were found in 4 of 6 adrenalectomized controls and 4 of 5 adrenalectomized cobalt-treated rats, when autopsied at the end of 65 days.

The accessory cortical tissue was made up primarily of zona fasiculata but in many instances part of the zona glomerulosa and rarely the zona reticularis were seen. In no case was there any evidence of accessory adrenal medullary tissue.

Experiment II

A. *Peripheral Blood.* The hematocrit, hemoglobin and erythrocyte count for all groups showed essentially the same change as in Experiment I, with the exception that the final erythrocyte counts in the adrenalectomized groups were farther above those for the controls than in Experiment I (Figs. 1 and 2). The MCV, MCHC and MCH values for the cobalt-treated intact animals fell below control values during the entire treatment period. This would indicate that the cell produced under cobalt stimulation is smaller and contains a lesser amount and concentration of hemoglobin than normal. The MCV and MCH values in the adrenalectomized animals with and without cobalt were significantly ($P < .01$) elevated at 20 days while the MCHC was relatively unchanged. The adrenalectomized control MCV and MCH values were significantly ($P < .01$) below control values from 30–60 days. The MCHC values for the cobalt-adrenalectomized group fell after 30 days to a low value as did cobalt-treated normal animals.

B. *Blood Volumes.* At the end of 60 days of treatment the red cell volume was significantly ($P < .01$) elevated in the cobalt-treated normal and adrenalectomized groups (Table 3). The adrenalectomized controls showed erythrocyte volumes at 60 days slightly ($P < .01$) above normal controls. Therefore, it seems reasonable to assume that the relatively normal hematocrit at 60 days in this group was the result of an increased plasma volume. The red cell volumes of the adrenalectomized cobalt-

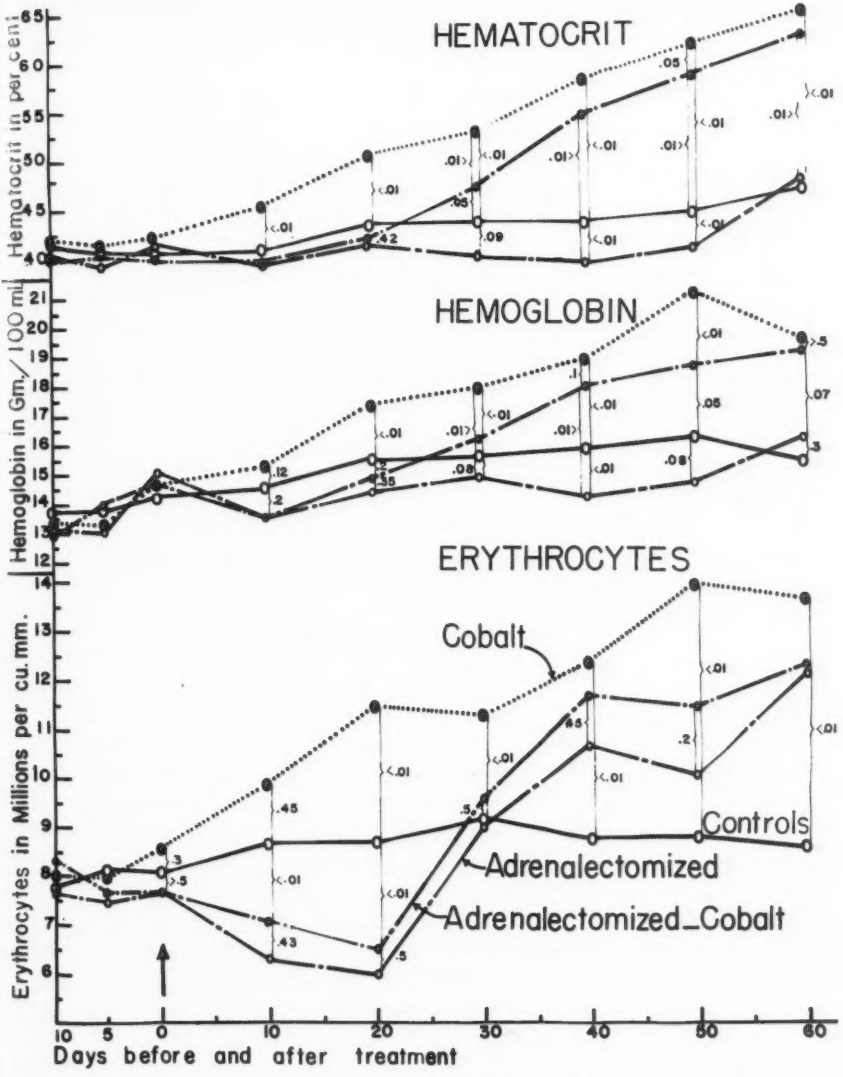

FIG. 1. Hematologic response of adrenalectomized rats treated with cobalt (Experiment II).

treated group were significantly ($P < .01$) elevated above those of the adrenalectomized controls.

C. *Adrenal Weights and Histology.* As in Experiment I the adrenal weights in the cobalt-treated group were significantly ($P < .01$) greater than normal controls (Table 2). The cobalt-treated normal animals also

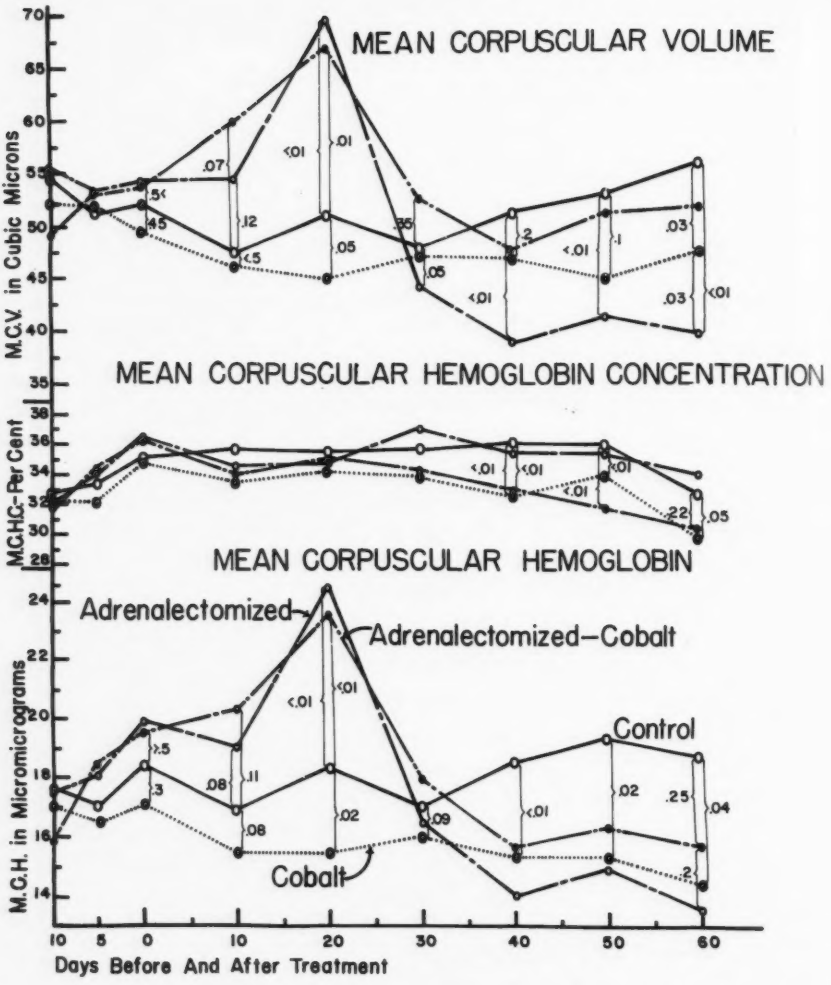

FIG. 2. Changes in MCV, MCH and MCHC in adrenalectomized and cobalt treated rats (Experiment II).

showed a moderate to marked depletion of lipid in the cortex. These changes were similar to but less intense than the type III response of the adrenal cortex described by Sayers *et al.* (16). Of the 4 animals in the adrenalectomized control group dying prior to 30 days, only one showed microscopic evidence of accessory adrenal tissue which consisted of zona fasiculata. None of the 3 adrenalectomized, cobalt-treated rats dying during the first 30 days possessed accessory adrenal tissue and none of these rats had responded to cobalt. One animal in the adrenalectomized cobalt

TABLE 3. THE EFFECTS OF ADRENALECTOMY, COBALT TREATMENT AND A COMBINATION OF ADRENALECTOMY AND COBALT ON THE TOTAL RED CELL VOLUME OF THE ADULT MALE RAT

Treatment	Weight (gm.)	No. animals	Hematocrit (per cent)	Total red cell volume (ml/100 gm. body wt.)
Control	387 ± 20.1	8	47.5 ± 1.8	2.42 ± 0.13
Cobalt Chloride*	315 ± 21.6	9	65.7 ± 2.9	4.99 ± 0.64
Adrenalectomized	345 ± 52.7	4	48.0 ± 1.4	2.80 ± 0.22
Adrenalectomized Cobalt Chloride	298 ± 4.6	3	63.3 ± 1.5	4.57 ± 0.45

* = Dosage was 2.5 mg. elemental cobalt/kg. daily for 60 days.
± = Standard Deviation.
P = "t" test of significance. Comparisons of total red cell volumes between groups were:
cobalt vs. controls—$P < .01$
adrenalectomy—cobalt vs. adrenalectomy—$P < .01$
adrenalectomy—cobalt vs. cobalt—$P = .20$
adrenalectomy vs. control—$P = .02$

group which died under anesthesia at 40 days showed an accessory adrenal on the left side in which all three zones of the cortex were well differentiated but no medullary tissue was present. All adrenalectomized animals with and without cobalt treatment possessed adrenals confirmed microscopically when autopsied at the end of 60 days. It is likely that the adrenalectomized rats surviving beyond 20–30 days depended upon the presence and functional activity of accessory cortical tissue.

Experiment III

Sham Operations. After 10, 22 and 30 days of cobalt treatment, rats subjected to a sham operation responded with a polycythemia at approximately the same rate as intact rats given cobalt. Twenty-two day hematocrit values for intact rats given cobalt and rats subjected to sham operation and given cobalt were 50.0 ± 3.3% and 49.6 ± 1.4%, respectively; the 30-day values were 54.2 ± 3.3% and 51.4 ± 4.1%. These differences were not significant. The hematocrit values were still elevated significantly 10 and 20 days after cobalt treatment was stopped in both the cobalt controls and the cobalt-sham operated group. Thirty days after stopping cobalt treatment the hematocrit values had returned to approximately their normal values.

This experiment proves that the procedures used in adrenalectomy are not in themselves sufficient to alter the response to cobalt during the first 30 days after operation.

DISCUSSION

The finding of a moderate anemia during the first 20 days after adrenalectomy is in agreement with the findings of Crafts (17) and Gordon (18). In Experiments I and II cobalt was ineffective in correcting the anemia occurring during the first 20–30 days after adrenalectomy. Thus, the polycy-

themic response to cobalt was, for the most part, absent during this period. Results from Experiment III indicate that the sham operation did not alter the polycythemic response to cobalt.

The interpretation of these experiments depends so intimately on the occurrence of accessory adrenals following adrenalectomy that the literature concerning this point should be cited. Jaffe (19) performed bilateral adrenalectomies on 90 young albino rats kept under good laboratory conditions, and found that 35% died within 30 days, 46% between 1 and 7 months, and the remaining 19% survived indefinitely. Accessory cortical tissue was reported in all survivors. MacFarland (20) and Gaunt (21) found that completely adrenalectomized rats would survive if cortical tissue had enough time to proliferate from fibroblast-like cells of the coelomic wall.

The duration of anemia for about 30 days after adrenalectomy could be due to the fact that it takes 30 days for the accessory cortical tissue to become functional. The rather slow decline in hematocrit after the removal of cobalt, ultimately reaching values which were within normal limits 30 days after cobalt injections were stopped, is in harmony with the 45–50 day life span of red cells reported for the normal rat (22).

The elevation of MCV and MCH 20 days after adrenalectomy is in close accord with the finding of Gordon *et al.* (18) indicating the presence of a macrocytic anemia. It is quite possible that the decrease of the MCV and MCH in both adrenalectomized groups between 30–60 days from relatively high values at 20 days is also related to the beginning function of the accessory cortical tissue. The decreased size (MCV) and hemoglobin content per cell in the cobalt-treated intact rats could have been due to the lack of sufficient dietary iron for absorption to meet the demand of accelerated hemopoiesis. Kato and Iob (23) found that the bone marrow and spleen of cobalt-treated rats contained less iron than did those of normal controls.

The weight gain was essentially the same in Experiment I and II for both the normal and adrenalectomized groups treated with cobalt. Therefore, it seems unlikely that a non-specific effect due to a difference in food intake could have affected the response of the adrenalectomized animals.

Despite the evidence for a direct stimulating effect of cobalt on the adrenal gland, it is obviously quite possible that a "permissive" action of the adrenocortical hormones is exerted on either the bone marrow or other endocrine organs responsible for the cobalt action. During the first 30 days after adrenalectomy metabolic processes normally carried out by the hematopoietic tissue may either proceed at a slow rate or else exhibit marked deficiencies rendering them incapable of responding to the cobalt stimulus.

Recent work has shown that cobalt-treated intact rats developed high levels of an erythropoietic factor in the plasma as measured by the incorporation of Fe^{59} into the erythrocyte of starved or hypophysectomized rats (24). Bilaterally nephrectomized rats failed to respond to an injection of

cobalt; while removal of adrenals, seven-eighths of the liver, spleen, pancreas, stomach, or intestines from the rat did not lessen the response to an injection of cobalt (25). In view of the lack of experimental detail in these reports by Jacobson *et al.* (24, 25) it seems too early to seek an explanation for the lack of correlation between our results and theirs with regard to the role of the adrenal in the response to cobalt.

Several lines of evidence support our hypothesis that the polycythemia induced by cobalt is the result of an increased secretion of hormones by the adrenal cortex. The adrenal glands in the cobalt-treated intact rats were significantly larger and contained less sudanophilic substance than untreated controls. Obviously this evidence alone does not prove that the adrenal gland is related to cobalt polycythemia because it is well known that many moderately toxic agents are capable of producing adrenal enlargement and lipid depletion (26). However, this does indicate that cobalt is exerting some direct or indirect stimulating influence on the adrenal gland and this is consistent with our hypothesis. It is also known that ACTH injections (27) cause an enlargement of the adrenal gland and an increase in erythrocytes, hemoglobin, hematocrit and total red cell volume in the rat (9) (28). The polycythemia of Cushing's disease and the response of the bone marrow to adrenocortical extract (10) and cortisone (12) (13) tend to establish a connection between the adrenal gland and hematopoiesis. Also of considerable interest is the finding that the direct addition of cortisone to guinea pig bone marrow *in vitro* induces a marked increase in the occurrence of mitosis amoung the erythroid cells (29). The effects of the adrenal hormonal factors, seen in other *in vitro* studies of bone marrow seem to be exerted principally on the basophilic nucleated erythrocytes and total eosinophile cells (30).

Depressed oxygen tension causes polycythemia and animals in which erythropoiesis has been stimulated in this manner have enlarged adrenals, increased adrenocortical activity (31) (32) and depletion of sudanophilic substance from the adrenal cortex. However, Piliero (33) concluded that the polycythemia of depressed oxygen tension was not inhibited by adrenalectomy in the rat.

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RELATIONSHIP OF LOW PROTEIN DIET AND ASCORBIC ACID IN ESTROGEN INACTIVATION BY LIVER

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ABSTRACT

It has been shown that an adequate protein intake is necessary for estrogen inactivation by the liver (13, 14). Since it has been reported that liver glutathione and ascorbic acid are decreased in either low protein or protein free diets (16, 17, 18), this investigation was carried out to determine if ascorbic acid and glutathione are involved in estrogen metabolism in the liver. This was studied by both *in vivo* and *in vitro* experiments using Sprague-Dawley female albino rats.

In the *in vivo* experiments adult rats were ovariectomized and implanted with a pellet of estrone according to the method of Burrill and Greene (19). The inactivation of the estrogen was followed by the vaginal smear method. After three weeks of anestrous smears, the animals were placed on an 8% casein diet and within 10 days the animals lost the ability to inactivate the estrogen. Beginning on the first day of the 11th week, glutathione was injected intraperitoneally daily for two weeks, followed by daily injections of ascorbic acid in addition to the glutathione for two more weeks. In a separate experiment ascorbic acid alone was injected for 8 days. The effect of glutathione was sporadic but when ascorbic acid was added, either with glutathione or alone, the animals regained completely the ability to inactivate estrone.

In the *in vitro* studies one group of adult female rats was maintained on the same 8% casein diet used in the *in vivo* experiments. A second group of rats was maintained on an adequate 21% casein diet in which there was an isocaloric substitution of the added casein for sucrose. After 2½–3 months the rats were sacrificed and the livers removed and sliced. The liver slices were incubated with estrone or estradiol, with and without ascorbic acid, for 1–1½ hours at 37° C. The amount of estrogen inactivation was measured by the bioassay method of Lauson *et al.* (22).The effect of diphosphopyridine nucleotide, glutathione and liver extract on the inactivation of estrone by liver slices was also studied.

The results of the *in vitro* experiments showed that liver slices from the 8% casein diet rats could not inactivate estrone even in the presence of ascorbic

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acid, diphosphopyridine nucleotide, glutathione or liver extract. However, the 21% casein diet rat livers inactivated 60% in 1½ hours. The data show that liver slices from the 8% casein diet rats could inactivate estradiol as efficiently as liver slices from 21 %casein diet rats.

IT HAS been demonstrated that the estrogens are inactivated by the liver of various animals, one known exception being the monkey (1, 2, 3, 4, 5, 6). The recognition of a relationship between the nutritional status of the animal and the ability of the liver to inactivate estrogen was initiated by Biskind and Biskind (7, 8) who observed that rats placed on a vitamin B-complex deficient diet lost the ability to inactivate intrasplenic implanted estrone pellets. Segaloff and Segaloff (9) and Singher *et al.* (10) found that thiamine and riboflavin were the members of the vitamin B-complex necessary for normal estrogen metabolism by the liver. Subsequently, these findings were reported by Drill and Pfeiffer (11) and by Jailer (12) to be due to the decreased food intake that accompanies an acute deficiency of these vitamins. It has further been shown by Jailer and Seaman (13) and by Vanderlinde and Westerfeld (14) that the inanition effect is due to a decreased protein intake rather than a caloric restriction. The importance of adequate protein in the diet was first indicated by Unna *et al.* (15) when they reported that liver slices from rats maintained on an 8% casein diet were unable to inactivate estradiol.

Liver glutathione and ascorbic acid are known to rapidly decrease in rats on a protein free diet (16, 17) and a low protein diet has been reported to cause a decreased synthesis of ascorbic acid in the white rat (18). Since the dietary level of protein apparently effects the liver levels of ascorbic acid and glutathione, it appeared that these two compounds might be involved in estrogen metabolism.

The purpose of this investigation was to determine if ascorbic acid and glutathione are involved in estrogen metabolism in the liver. The results of our experiments indicate that ascorbic acid may have a function in estrogen inactivation by rat liver.

METHODS

Sprague-Dawley female albino rats were used in all the experiments. *In Vivo Studies.* Adult rats weighing 150–225 gm. were ovariectomized and implanted with a pellet of estrone according to the method of Burrill and Greene (19). Following a control period of anestrous vaginal smears for 3 weeks during which time the animals were fed Purina dog chow *ad libitum*, the rats were placed on a low protein (8% casein) diet. The purified 8% casein diet was the same as that described by Westerfeld and Richert (20) except that sucrose was subtituted for glucose. Vaginal smears were taken daily until the experiment was terminated (T). Within an 8–10 week period after the start of the 8% casein diet the animals showed continuous positive vaginal smears due to their inability to inactivate estrone. It had been demonstrated previously by Vanderlinde and Westerfeld (14) that an 8% casein diet fed *ad libitum* to rats resulted in an inability to inactivate estrone absorbed from an estrone pellet. Beginning on the first day of the eleventh week 25 mg. glutathione (GSH) in 0.5 cc. physiological saline was injected intraperi-

orally daily for 2 weeks. This was then followed by daily injections of 50 mg. of ascorbic acid (AA) in addition to the glutathione for two more weeks. In a separate experiment ascorbic acid (AA) alone was injected for 8 days. The effect of glutathione and ascorbic acid on estrone inactivation by 8% casein diet rats was observed by the vaginal smear method.

In Vitro Studies

A. *Estrone Inactivation.* One group of adult female albino rats was maintained on the same 8% casein diet used in the *in vivo* experiments. A second group of rats was maintained on an adequate 21% casein diet. The 21% casein diet was the same as the 8% casein diet except for the isocaloric substitution of the added casein for sucrose. Both groups were allowed to eat *ad libitum* and to drink tap water *ad libitum.*

After 2½–3 months, the rats were sacrificed by a blow on the head followed by decapitation and exsanguination. The livers were immediately removed and cooled by placing in ice cold petri dishes containing filter paper moistened with phosphate buffer solution, pH 7.2. The livers were then sliced with a Stadie-Riggs microtome tissue slicer. Accurately weighed samples of liver slices (approximately 100 mg.) from each rat were incubated with 5 μg. estrone and with 5μg. estrone plus 2 mg. ascorbic acid, respectively. The estrone was dissolved in phosphate buffer, pH 7.2, by the method described by Lehninger and Scott (21). The final volume of each incubation tube was made up to 3 cc. with this phosphate buffer. The tubes were placed in a 37° C water bath and after allowing 15 minutes for the tubes to equilibrate, they were shaken for 1½ hours. The gas phase was air. The tubes were then removed from the bath and placed in boiling water for 10 minutes to inactivate the reaction mixture. After cooling, the contents of each tube were homogenized and diluted to 9 cc. with phosphate buffer, pH 7.2. The diluted homogenate of each tube was bioassayed for its estrogen content by the uterine weight method of Lauson *et al.* (22). The increase in uterine weight of immature 21-day old female rats was measured after they had received injections of aliquots of the homogenates corresponding to no more than 0.4 μg. estradiol or 2 μg. estrone, assuming no inactivation occurred. Lauson *et al.* (22) demonstrated that the increase in uterine weights due to the injection of estrogens into 22–23 day old Sprague-Dawley immature female rats was a reliable index of estrogen concentration. They showed, for example, that a linear relationship is obtained between the increase in uterine weight and the amount of estradiol injected (0.025 μg. to 0.4 μg.). Above 0.4 μg. estradiol the curve becomes asymptotic. For estrone a similar relationship exists for values of 1 μg. to 8 μg. estrone. Their results also showed that estriol was ⅛ as active as estradiol. The uterine response was found to vary with different experiments, resulting in either smaller or larger uterine weight responses, due to a change in the specificity of animals. However, the same linear relationships existed.

In order to test the reliability of this bioassay method with the animals used in our experiments we repeated the experiments of Lauson *et al.* (22) except that our estrogens were dissolved in phosphate buffer, pH 7.2, for injection into 22–23 day-old immature female Sprague-Dawley rats. Each group contained 6–8 rats. We confirmed the results of Lauson *et al.* (22) with respect to the uterine weight response to estradiol injections. However, we found that our rats were more sensitive to estrone than was reported by Lauson's group (22). In our experiments we found that a linear relationship was obtained with 1–3 μg. estrone rather than 1–8 μg.

In order to account for the animal specificity variation in uterine weight responses in the bioassay of our inactivation experiments, controls receiving standard solutions of the estrogen involved were run simultaneously with the inactivation homogenates.

By performing experiments in which no inactivation of the estrogen occurs, it has been possible to show complete recovery of the estrogen by injection of the homogenates

into immature female rats. If liver slices were heated in boiling water for 10 minutes before being added to the incubation medium or unneutralized ascorbic acid is added to the incubation tubes, lowering the pH of the incubation medium to 3.3–3.5, complete recovery of the estradiol was obtained by the biosasay method outlined above. It is felt therefore that this bioassay method is a reliable method for determining the amount of estrogen remaining in the inactivation medium.

The effects of several cofactors on estrone inactivation by liver slices from 8% casein diet rats were also studied. Using the same experimental procedure as outlined above, the effects of oxidized diphosphopyridine nucleotide (DPN), reduced diphosphopyridine nucleotide (DPNH), glutathione (GSH) and liver extract on the inability of liver slices from 8% casein diet rats to inactivate estrone were studied. The liver slices were incubated with 5 μg. estrone plus one of the following: 0.3 mg. DPNH, 0.3 mg. glutathione, 0.3 mg. DPN plus 0.3 mg. glutathione, 0.3 mg. DPNH plus 0.3 mg. glutathione or 2.0 mg. liver extract (Sigma). All the listed cofactors were dissolved in the phosphate buffer of Lehninger and Scott (21), pH 7.2. The total volume of each incubation tube was 3.0 cc. and the gas phase was air.

B. *Estradiol Inactivation.* In this study the same experimental procedure as described under estrone inactivation was followed except that the incubation time was for 1 hour rather than 1½ hours since it was known that 100 mg. of normal rat liver slices could inactivate 5 μg. of estradiol in 1 hour (21).

RESULTS

In Vivo Studies

The effect of glutathione (GSH) injections on estrone inactivation was sporadic since an irregular pattern of positive and negative smears was observed (Fig. 1, upper half). When ascorbic acid (AA) was added along with glutathione, 4 of the 6 low protein diet rats regained completely the ability to inactivate estrone. Even after the injections of glutathione and ascorbic acid were stopped, approximately three weeks elapsed before positive vaginal smears reappeared. Animals 5 and 6 were unable to inactivate estrone even when ascorbic acid was added. There are several possible explanations to account for this: (a) glutathione and ascorbic acid had no effect, (b) collateral circulation may have developed, (c) the estrone pellets were misplaced on implantation, and (d) an overwhelming amount of estrone was absorbed from the pellet.

When ascorbic acid (AA) alone was injected, it was observed that within two days after the first injection, negative smears were obtained from 7 of the 8 rats used with one rat showing borderline smears (Fig. 1, lower half). Once the injections were stopped, it was expected that the rats would again show continuous positive smears but instead an irregular pattern of positive and negative smears was obtained. This may have been due to residual ascorbic acid resulting from the large doses administered. It appeared from these *in vivo* experiments that ascorbic acid may counteract the effect of a low protein diet on estrogen inactivation. We therefore attempted to further study the possible role of ascorbic acid by *in vitro* experiments.

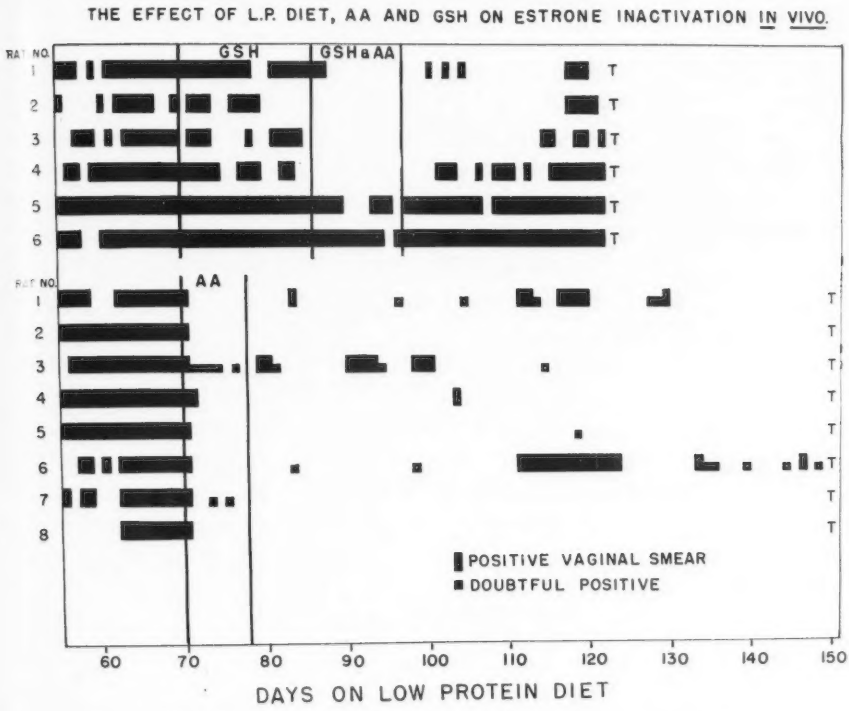

FIG. 1. The effect of low protein diet, ascorbic acid and glutathione on estrone inactivation *in vivo*.

In Vitro Studies

A. *Estrone Inactivation.* The data in Table 1 show that liver slices from 8% casein diet rats could not inactivate estrone but the 21% casein diet rat livers inactivated 60% in 1½ hours. In other similar experiments, normal rat livers inactivated as much as 95% of the estrone in 1½ hours. In contrast to our *in vivo* experiments, the addition of ascorbic acid *in vitro* did not result in a recovery of the ability of the 8% casein diet rat liver slices to inactivate estrone.

The addition of oxidized diphosphopyridine nucleotide (DPN), reduced diphosphopyridine nucleotide (DPNH), glutathione (GSH) and liver extract alone or in combination had no apparent effect on the inability of liver slices from low protein diet rats to inactivate estrone (Table 2). DPN has been shown to be required by normal rat liver for the inactivation of estrogen by DeMeio *et al.* (23). They demonstrated that if nicotinamide was not added to normal liver preparations before homogenization, the liver homogenates could inactivate estrogens approximately

TABLE 1. INACTIVATION OF ESTRONE BY RAT LIVER SLICES

Protein diet	No. of rats	Wt. of liver slices in mg.*	Liver slices incubated with†	Per cent inactivation
21% Casein	7	109.6 ± 2.68	Estrone	60.4
21% Casein	7	110.0 ± 2.24	Estrone + ascorbic acid	46.7
8% Casein	7	108.9 ± 1.22	Estrone	0
8% Casein	7	107.7 ± 2.19	Estrone + ascorbic acid	0

* All values are mean ± Standard Error.
† Incubation tubes contained either 5 μg. estrone or 5 μg. estrone plus 2 mg. ascorbic acid.

50% as efficiently as liver slices. The liver homogenates could inactivate estrogens as efficiently as liver slices if DPN was added to the homogenates. The destruction of DPN in the process of homogenizing liver tissue is prevented by the addition of nicotinamide prior to homogenization.

B. *Estradiol Inactivation.* The data of Table 3 show that liver slices from the 8% casein diet rats could inactivate estradiol almost as efficiently as liver slices from 21% casein diet rats. This is in contrast to the work of Unna *et al.* (14) who reported that liver slices from rats on an 8% casein diet could not inactivate estradiol. Several repetitions of this experiment consistently showd that liver slices from the 8% casein diet rats could inactivate estradiol as efficiently as normal liver slices could. Since liver slices from the 8% casein diet rats could inactivate estradiol, it was not possible to show what effect ascorbic acid might have on the *in vitro* inactivation of estradiol.

The inactivation of estradiol by both the 21% and 8% casein diet rats does not appear to be simply a conversion to the less active estrone. This was shown by injecting into recipient rats aliquots of incubation tube homogenates equivalent to 2 μg. estradiol, assuming that no inactivation occurred. This quantity did not cause any increase in uterine weights. If estradiol had been converted to estrone, a response in uterine weight equivalent to at least 2 μg. estrone should have been observed. Preliminary studies with pure estrone standards showd that a 66% increase in uterine weight over the controls is obtained with 2 μg. estrone. This experiment, then, would indicate that estradiol was not converted to estrone.

TABLE 2. EFFECT OF COFACTORS ON INACTIVATION OF ESTRONE BY RAT LIVER SLICES

Protein diet	No. of rats	Wt. of liver slices in mg*	liver slices incubated with†	Per cent inactivation
21% Casein	8	108.3 ± 0.734	Estrone	95.1
8% Casein	4	105.0 ± 1.73	Estrone	29.5
8% Casein	4	103.5 ± 0.866	Estrone + DPN	34.5
8% Casein	4	104.0 ± 2.32	Estrone + DPNH	16.3
8% Casein	4	102.5 ± 1.44	Estrone + GSH	28.8
8% Casein	4	103.5 ± 2.06	Estrone + DPN + GSH	24.5
8% Casein	4	102.0 ± 0.656	Estrone + DPNH + GSH	27.6
8% Casein	4	106.0 ± 2.32	Estrone + Liver extract	22.1

* All values are mean ± Standard Error.
† 5 μg. estrone, 2 mg. liver extract and 0.3 mg. DPN, DPNH and GSH were used. When GSH was incubated along with either DPN or DPNH, 0.3 mg. of each compound were used.

TABLE 3. INACTIVATION OF ESTRADIOL BY RAT LIVER SLICES

Protein diet	No. of rats	Wt. of liver slices in mg.*	Liver slices incubated with†	Per cent inactivation
21% Casein	10	101.4 ± 1.18	Estradiol	97
21% Casein	10	101.3 ± 1.33	Estradiol + ascorbic acid	97
8% Casein	9	102.8 ± 2.39	Estradiol	92
8% Casein	9	103.1 ± 1.34	Estradiol + ascorbic acid	95

* All values are mean ± Standard Error.
† Incubation tubes contained either 5 μg. estradiol or 5 μg. estradiol plus 2 mg. ascorbic acid.

DISCUSSION

Although ascorbic acid injections resulted in the ability of 8% casein diet rats to inactivate intrasplenic pellets of estrone, it has not been possible to show any *in vitro* ascrobic acid enhancing effect with liver slices from low protein diet rats. It is difficult to explain this difference. It is possible that we are dealing with a permeability phenomenon which is not overcome in our experimental conditions. DPN, DPNH, glutathione and liver extract also failed to effect the inactivation of estrone by low protein diet rat liver slices. It may also be that the inability of liver slices from low protein diet rats to inactivate estrone is not due to the loss of a cofactor, at least not DPN, glutathione or a factor in liver extract, but rather to the inability to synthesize the enzyme catalyzing this inactiva; tion. Possibly the administration of ascorbic acid to the intact rat indirectly stimulates the synthesis of the enzyme. Since liver slices from low protein diet rats apparently do not contain a sufficient quantity of the inactivating enzyme, ascorbic acid would have no effect on liver slice inactivating ability since one of its functions may be to influence protein synthesis. However, the authors do not know of any evidence indicating that ascorbic acid is involved in protein synthesis.

Unna *et al.* (15) reported that liver slices from rats maintained on an 8% casein diet for 87–97 days could not inactivate estradiol. The results reported in this paper, however, show that liver slices from 8% casein diet rats inactivate estradiol as efficiently as normal liver slices do. The reason for these conflicting results is not apparent. It is of interest to note that Unna *et al.* (15) reported that liver slices from 8% casein diet rats receiving a daily supplement of methionine were able to inactivate estradiol.

The observation that liver slices from low protein diet rats could inactivate estradiol but not estrone is also worthy of discussion. It is generally accepted that in its metabolism, estradiol is converted reversibly to estrone which may then be further metabolized irreversibly to estriol. The data reported in the present paper suggests that the inactivation of the estrogens does not allow this sequence. For example, if the pathway of estradiol inactivation goes through estrone to estriol to further oxidation products, then low protein diet rats should be able to inactivate estrone

as well as estradiol. However, since it was observed that estrone was not inactivated by low protein diet rat liver slices, but estradiol was, it appears that possibly estrone is not an intermediate in estradiol inactivation. The normal inactivation pathway of estradiol may be its conversion to either estriol or other metabolic products but the inactivation of estrone may first require its conversion to estradiol. If this is true, then the enzyme responsible for the conversion of estrone to estradiol may be the one affected by the low protein diet. These low protein diet rats would then be unable to convert estrone to estradiol, accounting for the fact that liver slices from low protein diet rats can inactivate estradiol but not estrone. Heller (4) suggested many years ago that the conversion of estrone to estradiol may be the initial step in estrone inactivation.

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THE EFFECTS OF PANTOTHENIC ACID DEFICIENCY ON THE SECRETION OF CORTICOSTEROIDS BY THE ALBINO RAT[1]

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ABSTRACT

Pantothenic acid deficiency has been produced in mature rats by feeding a diet free of pantothenic acid and supplemented with ω-methylpantothenic acid (calcium salt). Adrenal venous blood was collected by cannulation of the left renal vein and the production of individual steroids of normal and deficient rats was estimated. The production of corticosterone, the predominant corticosteroid from the rat adrenal, was significantly reduced by the vitamin deficiency. The reduction was not due to a non-specific, toxic effect of ω-methylpantothenate. The results indicate that pantothenic acid is specifically required for normal corticosteroid production by the adrenal of the albino rat.

CONSIDERABLE indirect evidence has been presented to implicate pantothenic acid as an essential substance for normal function of the adrenal cortex in experimental animals (1–5). Deficiency of this vitamin has been developed in weanling rats (2), and the establishment of ω-methylpantothenic acid as a metabolic antagonist to pantothenic acid (6) has permitted the ready development of the deficiency in animals and in human subjects (7). In the latter, the clinical state and the responses to physiological testing all indicated the establishment of adrenal cortical deficiency. A direct study of corticosteroid production was made by Eisenstein (8) who developed the deficiency in weanling rats, incubated the adrenal glands *in vitro* in an artificial medium and determined the amount of steroids produced. He reported the production of steroids to be significantly reduced by the adrenals of deficient animals. The investigation reported below was conducted in an attempt further to evaluate the effect of pantothenic acid deficiency on the production of corticosteroids

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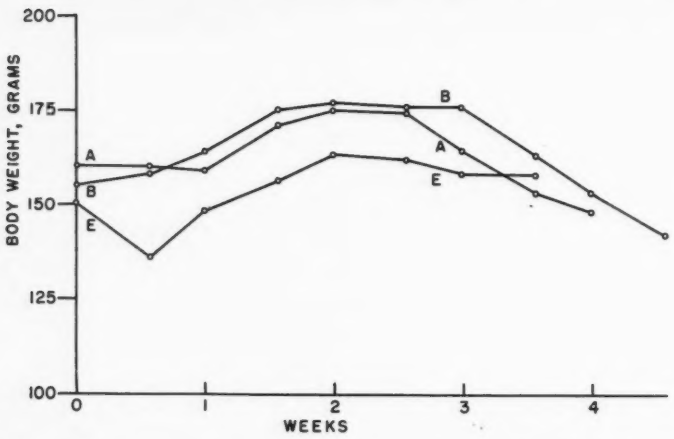

FIG 1. Examples of growth curves of animals on various diets. Letters refer to groups. Group A received synthetic diet containing a complete vitamin supplement. Group B received a diet identical to that of Group A but lacking pantothenic acid. On the 10th or 11th day of feeding 0.5 gm. of calcium ω-methylpantothenate per 100 gm. of diet was added. Group A was pair-fed to Group B. Group E received 6.5 gm. per day of the complete diets to which 0.5 gm. of ω-methylpantothenate per 100 gm. was added from the outset.

by separation and quantification of these compounds in adrenal venous blood of the albino rat.

METHODS

Male, albino rats of 150–250 gm. body weight were paired and placed upon either a complete,[4] synthetic diet or a diet identical in all respects except for the lack of pantothenic acid. The intake of the animals on the deficient diet was determined each day and on the following day the control animal of the pair received an equal amount of the complete diet. On the 10th or 11th day calcium ω-methypantothenate[5] was added to the deficient diet in the amount of 0.5 gm. per 100 gm. diet (6–9). Typical weight curves for animals on the various feeding regimes are presented in Figure 1.

When the animals were considered to have developed the nutritional deficiency, as judged by gross symptoms and body weight loss, cannulation was performed for the collection of adrenal venous blood. The first attempts were made on animals which had been on the diet for 28 days and which had been fasted overnight. This procedure resulted in death of many of the rats, presumably as the result of the overnight fast, or in their death in the early stages of the cannulation. Consequently, the final procedure adopted was that of operation on the 24th to the 26th day of the experimental diet and without an overnight fast.

The rats were anesthetized with ether, and a flank incision was made to expose the left kidney and renal vein, the left adrenal gland and its vein from the gland to its point

[4] The diets were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[5] Calcium ω-methylpantothenate, obtained from the California Foundation for Biochemical Research, Los Angeles, California, assayed 78 to 95% pure.

of entry into the renal vein. A ligature was placed around the renal vein between its confluence with the inferior vena cava and the adrenal vein. A second ligature was placed around the renal vein between the adrenal vein and the hilus of the kidney. At this point in the operation 0.2 ml. of heparin solution (5 mg. heparin per ml. of 0.9% sodium chloride solution) was administered via a tail vein. A longitudinal incision was then made in the renal vein about 5 mm. from the hilus of the kidney, and a polyethylene cannula (inside diameter 0.015 in., outside diameter 0.043 in.) was inserted in the vein. When flow of blood had been established, the cannula was securely tied in place and was then stablilized by a ligature placed through the kidney and tied around the cannula. The second ligature was then tied and a segment of the renal vein receiving the flow from the adrenal vein was thus isolated. The blood was allowed to flow into a small centrifuge tube immersed in an ice bath until the demise of the animal, or until its physical state indicated that its end was imminent. A second dose of 0.1 ml. of heparin solution was administered at about 20 to 30 min. after blood collection was begun. At the end of the experiment the animal was weighed, its adrenal glands were removed, weighed and fixed for histochemical examination.[6]

The extraction of corticosteroids by a method involving dialysis (10), solvent extraction and chromatography (11) is described by Reif and Longwell (17). Individual steroids were quantified (17) by measuring light absorption at 240 mμ, as well as by their reaction with blue tetrazolium (12) or by the Zimmerman reaction as modified by Vestergaard (13). The efficacy of all procedures was checked with pure, crystalline steroids[7] (17).

In the early stages of this investigation an attempt was made to conduct the study in the more classical fashion by producing the deficiency in weanling rats without the use of a vitamin antagonist. This was successfully accomplished, and it was possible to carry out the cannulation of the renal vein. However, the young rats were unable to withstand the trauma of the operation for sufficient time to collect an adequate amount of blood. This part of the investigation was, therefore, abandoned.

RESULTS

The results of Experiments A to G, inclusive, are summarized in Table 1. Three constantly occurring areas appeared on the chromatograms (Fig. 2). Two of these corresponded to previously reported steroids, corticosterone (Kendall's Compound B) and Bush's (11) Compound X. The third substance, herein designated Compound Y, was more polar than Compound B. The following R_f values were determined (17): Compound Y, 0.62; Compound B, 0.73; Compound X, 0.86. Each value is the mean of 13 or 14 determinations of each compound. In these experiments the direct, visual observation of Compounds E and F in ultraviolet light was frequently uncertain. Sections cut from the paper to include the R_f areas determined with standard Compounds E and F always showed a determinate quantity of α,β-unsaturated substance. This area also contained

[6] Histochemical examinations were made by Dr. T. L. Chiffelle, Pathologist, The Lovelace Foundation.

[7] Gifts of the following steroids are gratefully acknowledged: Compound B by Dr. H. L. Mason, Mayo Foundation, Rochester, Minnesota; Compounds F and E by Drs. Elmer Alpert and F. K. Heath, Merck and Co., Inc., Rahway, New Jersey; 11β-hydroxy-Δ^4-androstene-3,17-dione by Dr. N. L. Heminway, Schering Corporation, Bloomfield, New Jersey. Additional steroid standards were obtained from U.S.P. Reference Standards, 46 Park Avenue, New York 16, New York.

whatever aldosterone was present in the extracts (14), and the material obtained from it is designed "E-F" in Table 1.

The values given in Table 1 are corrected for per cent recovery by the separathion method as determined with pure steroids. The actual quantities found (17) were 50% lower than those recorded in Table 1. The

TABLE 1. SUMMARY OF RESULTS FOR EXTRACTION OF STEROIDS FROM ADRENAL VEIN BLOOD OF NORMAL AND PANTOTHENIC ACID DEFICIENT RATS

Group*	Description	No. rats§	Ml. blood collected per rat per hr.	Steroids† corrected for 50% recovery									
				μg. steroid per ml. blood					μg. steroid per adrenal per kg. rat per hour				
				"E-F"	Y	B	X	Total	"E-F"	Y	B	X	Total
A	controls	7	2.7	1.3	7.1	18.5 ±8.0‡	7.3	34.2	23	120	315 ±146‡	123	581
B	deficients	11	2.4	0.5	4.8	10.8 ±3.5‡	4.6	20.7	8	79	178 ± 78‡	76	341
E	toxicity controls	5	2.6	0.9	8.3	24.2	9.5	42.9	13	128	374	147	662
F	controls +ACTH	5	2.9	0.7	6.9	23.8	6.5	37.9	15	141	489	133	778
G	deficients ACTH	4	2.0	0.6	1.9	9.1	0.0	11.6	9	28	135	0	172

* Group A received the complete diet and was pair-fed to Group B.
Group B received the pantothenic acid deficient diet which was supplemented with 0.5 gm. ω-methylpantothenate per 100 gm. of diet.
Group E received a complete diet with 11.6 mg. of pantothenic acid and 0.5 gm. ω-methylpantothenate per 100 gm. of diet.
Groups F and G were prepared nutritionally as Groups A and B, but received 0.1 ml. ACTH gel 24 and 4 hours before the cannulation. Armour's Acthar gel, containing 40 Armour units per ml., was used.
† "E-F", hydrocortisone, cortisone, aldosterone; Y, unknown compound; B, corticosterone (Kendall's Compound B); X, Bush's (11) Compound X.
‡ Standard deviation of individual chemical separations.
§ Individual chemical separations as follows: Group A, 4; B, 4; E, 2; F, 2; G, 2.
The combined ratios for all groups considering corticosterone as 1.00, for the various compounds were:
"E-F" =0.05, Y =0.36, B =1.00, X =0.33.

results show that the animals of Group A, the complete diet group, produced 315 μg. of corticosterone per adrenal per kg. per hr. In comparison, Group B, the pantothenic acid deficient group, secreted only 178 μg. per adrenal per kg. per hr. This was the steroid produced in greatest amount by both groups. The effect of the pantothenic acid deficiency is indicated by the 44% reduction in the output of Compound B. Furthermore, the production of the other compounds by Group B as compared to Group A shows a 65% decrease for Compounds "E-F," 38% for Compound X and 34% for Compound Y. Similar reductions were reported by Eisenstein (8).

Group E is composed of rats which received the complete diet with the addition of calcium pantothenate to a total concentration of 11.6 mg., and 500 mg. of ω-methylpantothenic acid per 100 gm. of diet (6). The food intake was restricted to the average daily intake of the controls, Group A, or 6.5 gm. per day. This gave a total intake over the course of the experiment of more ω-methylpantothenate than had been received by Group B, the deficiency group. The results show that the secretion of all four measured substances was of the same order, but somewhat higher than that of Group A, the control group. Thus it is evident that the effects of the vita-

min analogue observed in Group B represent a specific action of ω-methylpantothenate and not a general toxic effect.

Groups F and G were prepared nutritionally in the same manner as Groups A and B, Group F being the control, complete diet group and Group G the deficiency group. Each animal received 0.1 ml. (4.0 units) of adrenocorticotrophic hormone (ACTH, Armour's Acthar gel) intramuscularly 24 hr. and 4 hr. preceding the cannulation. Whereas the mean production of Compound B was 55% greater in Group F than in Group A (Table 1), the large standard deviation of the mean in the control group precludes unequivocal conclusion that an ACTH effect was evident under these experimental conditions. Group G, the deficiency group, showed no effect by ACTH. This group differed from all the others, also, in that the amount of Compound X was too small to permit acceptable quantification.

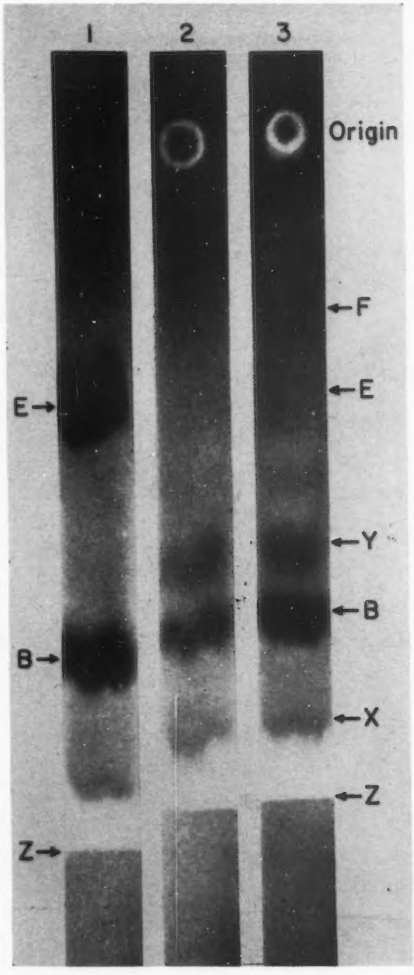

FIG. 2. Chromatograms of corticosteroids from adrenal vein blood by the Bush (11) method. Strip 1, standards; strip 2, animals on a complete diet; strip 3, animals on a deficiency diet. Y = unknown compound; B = corticosterone; X = Compound X of Bush (11); E = cortisone; Z = solvent front. Photographed (17) under UV light,* suspended 10 in. above and 10 in. to the side, using approximately a ten minute exposure at F-5.6, with panchromatic film (ASA 200) and Wratten filter No. 8.

* Spot quartz lamp with Wood's filter, Ultra Violet Products, Inc., 145 Pasadena Ave., South Pasadena, California.

Many of the chemical analyses in the above groups were performed on pooled blood from 2, 3 or 4 rats. When these results were subjected to statistical comparison based on each individual chemical separation, they failed to show significance. Therefore, two additional groups were examined in which the steroid analyses were performed on specimens from individual

TABLE 2. STEROID CONTENT OF ADRENAL VENOUS BLOOD FROM NORMAL AND PANTOTHENIC ACID DEFICIENT RATS*

	Group H Normal diet†	Group I Pantothenic acid deficient diet	Calculation of significance
Silber-Porter Reaction			
μg. Steroid per ml. blood	5.1± 2.4	1.4± 1.4	$P<0.01$
μg./kg./adrenal/hr.	71.0± 32.4	18.2± 18.5	$P<0.01$
Blue Tetrazolium Reaction			
μg. Steroid per ml. blood	34.5± 13.9	12.3± 7.3	$P<0.01$
μg./kg./adrenal/hr.	492 ±223	154 ±120	$P<0.01$
UV Absorption at 240 mμ			
μg. Steroid per ml. blood	35.7± 9.2	20.0± 7.6	$P<0.01$
μg./kg./adrenal/hr.	512 ±326	246 ±156	$0.05>P>0.02$

* Each group contained nine rats. The blood samples were analyzed individually. All values are corrected for 90 per cent recovery (17).

† Mean body weights: Group H, 207 gm.; Group I, 199 gm. Mean Volume of blood collected: Group H, 4.0 ml.; Group I, 2.8 ml. Mean time of collection: Group H, 79 minutes; Group I, 73 minutes.

animals. The results are recorded in Table 2. As in the other experiments, the steroids were separated from whole blood by dialysis against 40% methanol, methylene chloride extraction and purfication. After solution in 10 ml. ethanol, aliquots of the extract were analyzed by the Porter-Silber (19) reaction, the blue tetrazolium reaction and by measurement of light absorption at 240 mμ. The results, in terms of total steroid produced, were in good agreement with those obtained after paper chromatography. Statistically, the difference between the two groups is highly significant (Table 2), and gives ample evidence that pantothenic acid is essential to the production of corticosteroids as evaluated by this experimental approach.

Compound Y was compared to several crystalline steroids in order to determine its identity. The results of these experiments are described in the report of the methods used (17).

DISCUSSION

As stated above, the analysis of pooled specimens in Groups A and B reduced the number of experiments to a point at which statistical evaluation failed to give unequivocal results, in spite of a reduction of 44% in the mean secretion of Compound B by the deficiency group. However, when blood from individual animals was analyzed without chromatographic separation, comparison of the control and deficiency group (Table 2) revealed the following significant differences: Porter-Silber reaction, $P<0.01$; blue tetrazolium reaction, $P<0.01$; spectrophotometric analysis at 240 mμ, $P<0.05$.

Inasmuch as the control animals of Groups A and H were pair-fed to their respective deficiency group, the intake level of calories and vitamins other than pantothenic acid were comparable, and the diminished output

of Compound B seems attributable to pantothenic acid deficiency. Substantiation of this conclusion is found in the full secretion of Compound B in Group E, in which ω-methylpantothenic acid was fed together with an adequate supply of pantothenic acid. This latter observation appears to rule out any possible non-specific, toxic effect of the antivitamin. These results confirm, by direct steroid measurement, the earlier experiments which, by indirect chemical, physiological and histological methods, indicated that pantothenic acid deficiency reduced the production of adrenal cortical hormones.

In comparison with Group A, the complete diet group, administration of ACTH gel resulted in an increase of corticosterone production per adrenal per kg. per hr. by the adrenal of rats of Group F on the complete diet, but not by those of Group G, which were deficient in pantothenic acid. There was also an increase in its concentration per ml. of blood, 18.5 compared to 23.8 μg. per ml. However, when Group E, which received ω-methylpantothenate together with an adequate intake of pantothenic acid, is used for comparison the blood levels are substantially equal. The ACTH has an apparent effect here only when the results are calculated in terms of μg. per adrenal per kg. per hr., a frequently used method of evaluation (14, 15). The mean values for corticosterone production indicate that stimulation by ACTH occurred in the normal group and did not occur in the pantothenic acid deficient group, but the large standard deviation of the mean in the control group to which they are compared precludes unequivocal evaluation of ACTH effects under these experimental conditions.

The results reported above are compared to those of other investigators in Table 3. Bush (11) described the separation of Compounds B, F and X from rat blood. Bush also reported the Compound F/Compound B ratio to be less than 0.05. In the present experiments this ratio ranged from 0.03 to 0.07, a relationship of similar order (Table 1). The ratio for Compounds Y/B varied between 0.21 and 0.45 and that for Compounds X/B between 0.0 and 0.45. The nature of the experiment precludes attachment of significance to these variations.

TABLE 3. COMPARISON OF DATA ON CORTICOSTEROID CONTENT IN THE ADRENAL VENOUS BLOOD OF NORMAL RATS

Authors	No. rats	Sex	Mean wt., gm.	μg. B* per ml. adrenal blood	μg. steroid* per adrenal per kg. rat per hr.						Corrected for recovery
					"E-F"	Ald.	Y	B	X	Total	
Bush (11)	11	M	—	—	—	—	—	—	—	166.5–499.5	?
Singer & Stack-Dunne (14)	≃50	M(F)	≃250	10	—	0.31	—	150	—	—	no
Rosenman, *et al.* (15)	10	M	238	11.6†	14	—	—	160	—	—	no (?)
Beigelman, *et al.* (18)	—	M	—	4.1–6.7	—	—	—	72–136	—	—	—
Longwell, *et al.*	7	M	158	18.5‡	23	—	120	315	123	581	yes

* For description, see footnote to Table 2.
† Calculated from the value for plasma by division by 2.
‡ The uncorrected values for Compound B are 9.2 μg. per ml. and 158 μg. per adrenal per kg. per hour.

The reported concentrations of corticosterone in rat adrenal venous blood are 11.6 μg. per ml. (15), approximately 10 μg. per ml. (14) and 6.7 μg. per ml (18, highest value under ether anesthesia). Data for blood concentrations were not given by Bush (11). The present experiments record a value of 18.5 μg. per ml. in Group A, the normal controls. This figure is corrected for per cent recovery from a value of 9.2 μg. per ml. actually measured. Thus, there is good agreement between the reported values.

Evaluation of these data in terms of a presumably "normal" rate of secretion must be made with consideration of the ether anesthesia and operative trauma, which have a marked stressing effect (16, 18).

Bush (11) reported the occasional occurrence of Compound A in rat adrenal venous blood. A compound was observed in the present experiments in the position characteristic of Compound A only on occasional chromatograms (17), but it was not constantly present and no quantitative determination was attempted.

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EXTRACTION OF CORTICOSTEROIDS FROM ADRENAL VEIN BLOOD OF RATS AND RABBITS[1]

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ABSTRACT

A solvent extraction method is described that is simple and avoids use of evaporation at elevated temperatures. The extraction procedure gives 90% recovery of total steroids from whole blood. Extraction and chromatography give 50% recovery of individual steroids from whole blood. The method has been applied to the identification of steroids in rat adrenal vein blood. Some reactions of a steroid slightly more polar than corticosterone are described; the indication is that it has the basic configuration of 11-desoxy-17-hydroxycorticosterone, with some additional molecular modification. Evidence is given to support the presence of 11-dehydrocorticosterone, and the presence of corticosterone has been confirmed beyond reasonable doubt. The method has also been applied to the quantification of steroids in rabbit adrenal vein blood. Total Δ^4-3-ketosteroid output averaged 22 μg./ml. blood or 64 μg./adrenal/kg./hr. over a collection period of three to four hours. Steroid output per unit volume of blood was relatively constant throughout the collection period.

THE very existence of many different methods for isolation of adrenal cortical hormones from blood or plasma is an indication that the search for a method embodying all of the most important criteria of a successful method has not ended. These criteria include purity of isolated steroids, absence of artifact formation, high yield, good reproducibility, simplicity and rapidity of the method.

Present opinion favors the view that either whole blood or plasma may be employed for the extraction of adrenal cortical hormones, since adrenal steroids are present exclusively in plasma, and will not appreciably penetrate the formed elements of blood if the plasma is separated within an hour after collection (19). As a preliminary step that simplifies the subsequent purification process by removing much of the fat and cholesterol present, the dialysis method of Zaffaroni and Burton (34) may be used, or the modification of this process devised by Axelrod and Zaffaroni (1) or Lombardo *et al.* (16). As an alternative, direct extraction of whole blood or plasma with organic solvents such as ethyl acetate, chloroform, methanol,

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ether-chloroform, or methylene chloride have been employed. The extract is generally washed with dilute alkali to extract unknown formaldehydogenic substances that react atypically on oxidation with periodic acid, as well as known acidic substances such as fatty acids and bile acids (17). Traces of alkali are then removed by washing with a small volume of water. In order to eliminate nonsteroidal pigments, partitioning between 70% ethanol and petroleum ether is employed, or less frequently charcoal washing (17) or acetylation of the steroid (11). Alternatively, one or more preliminary chromatographic steps may be used (4, 9, 14, 21, 34). All the above cited authors as well as Nelson and Samuels (20), Bongiovanni and Eberlein (5), Rauschkolb *et al.* (23), Sweat (27), and Weichselbaum *et al.* (31) used chromatography as the final step of the purification procedure.

The present report describes a purification procedure characterized by a minimum number of steps. Procedures such as repeated evaporation at elevated temperatures, to permit solution of the steroids in another solvent, only cause possible loss or alteration of the steroids and have been eliminated. Recovery figures for each step of the procedure have been determined. The procedure has been used to attempt isolation, quantification and identification of certain adrenal cortical hormones in rat adrenal venous blood, and has also been applied to rabbit adrenal vein blood.

MATERIALS AND METHODS

The procedure for extraction of corticosteroids employs a standard method for the dialysis of whole blood (34), followed by an extraction method designed to leave the steroids sufficiently purified either for direct chemical determination or for further resolution by a well-used paper chromatographic method (7). All solvents used are certified chemicals of highest purity and are redistilled before use. Ethanol and methanol are distilled from 10 gm. 2,4-dinitrophenylhydrazine per liter, tail fractions are discarded, and the middle fraction is redistilled.

Whole heparinized blood is collected in vessels immersed in ice water. Immediately after collection, 1 volume of blood is diluted with 1 volume of water and 1 volume of absolute methanol, transferred to dialysis tubing (Arthur H. Thomas) and gently mixed by repeated inversion. The mixture is dialysed at room temperature for eight days with daily changes of the dialysate, which consists of an equal volume of 40% methanol (34). Each day after removal of the dialysate, the dialysis sack contents are mixed by inversion. The pooled dialysate is washed in a separating funnel with three successive portions of petroleum ether, each one-fifth the total volume of the dialysate. Next 11 ml. 1 *M* sodium carbonate are added for each 100 ml. of dialysate and the alkalinized aqueous methanol is extracted three times each with one-eighth its volume or with 15 ml. of methylene chloride, whichever is the larger volume. The methylene chloride extract is washed with one-quarter its volume of approximately 0.01N acetic acid, run into an evaporating dish equipped with a centerwell, and allowed to evaporate to dryness at room temperature. The residue is taken up in ½ ml. methylene chloride, spotted on a Whatman No. 1 filter strip, and the transfer completed with three successive portions of ½ ml. methylene chloride; alternatively, methyl alcohol may be used for the transfer. The spot is now chromatographed in the Bush (6, 7) toluene-methanol-water (4:3:1) system at 36–37° C, following overnight equilibration.

Further relevant details of the above procedure are as follows. All glassware is rinsed with pure methanol before use and the stopcocks of the separatory funnels are wetted with 40% methanol. Sixty very gentle shaking movements are used in the first washing or extraction of the dialysate, but more vigorous shaking can be employed in later separations, as long as it is controlled to avoid emulsions that require longer than 5 to 10 minutes for complete resolution. The Pyrex evaporating dish was specially constructed with a centerwell[3] holding ½ to 1 ml. and is illustrated in Figure 1. In this dish, 100 ml. methylene chloride will evaporate at room temperature if left standing overnight. The filter paper for chromatography is bought in a 1 in. wide roll. Lengths of 50 cm. are cleaned by successive overnight immersion in two cylinders, each containing 1 liter of pure methanol, and subsequently drying in air. To speed plating, a small jet of filtered nitrogen is directed against the underside of the spot, but no heating device is employed. After chromatography (7), the chromatogram strips are dried in air for an hour and routinely photographed under strong ultraviolet (UV) light. The dark spots and boundary are now marked with pencil under UV for determination of R_f values and then the paper strip is cut into sections of equal length (in the range 2.5 to 3.5 cm.), with each section containing one complete spot. Each section is shredded into a test tube and left to stand for two hours after addition of 5 ml. absolute ethanol, using a cork covered with aluminum foil as stopper. Blank values are determined by elution of sections of equivalent R_f values, taken from blank paper strips, which were chromatographed along with the experimental strips. The eluates may be used directly for determination of optical density at 240 mμ, or aliquots may be subjected to suitable chemical determinations (8, 29). Alternatively, use of the Bush chromatogram (7) has the advantage that all the steroids extracted are obtained spread out on a single strip of paper. If desired, selected areas may then be eluted and rechromatographed in other solvent systems in order to obtain further resolution.

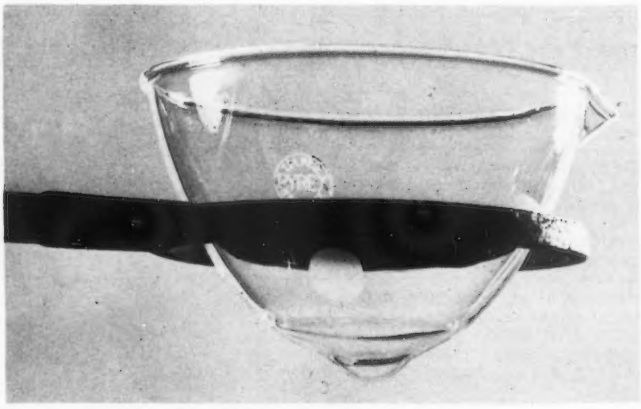

FIG. 1. Evaporating dish with centerwell.

RECOVERIES

The present procedure can be subdivided into five separate steps, each of which entails losses that are reported below.

[3] Arno P. Roensch, S. W. Scientific Glass Co., Route 1, Box 201-A, Santa Fe, New Mexico.

Dialysis: Table 1 gives the results obtained in a typical recovery experiment. Because it has been shown that in dialysis the recovery of steroids from whole blood is less than the recovery from pure methanol (33), recovery experiments using both whole blood and pure methanol were undertaken. Sixty-one μg. corticosterone were added to 10 ml. of a mixture of equal parts peripheral blood (from fasted rats), water and methanol, dialysed and extracted by the solvent procedure described above. After evaporation of the methylene chloride, the residue was not chromatographed but was taken up in 5 ml. ethanol for determination of the optical density at 240 mμ. Then a 3 ml. aliquot was used for the blue tetrazolium (BT) procedure, giving another figure for the recovery of the added corticosterone. It is seen that the recoveries for C experimentals in Table 1, respectively 88.6 and 90.7% for the OD_{240} and BT values, agree well. Since C experimentals underwent both dialysis and extraction, B controls were run to obtain a recovery figure for the extraction procedure alone. From these two values, the recovery for the dialysis procedure alone is readily calculated as 98.6%.

TABLE 1. RECOVERIES OBTAINED WITH THE DIALYSIS AND SOLVENT EXTRACTION PROCEDURES

Experiment (No. of determinations in parentheses)	OD at 240 mμ	OD for BT* test	Per cent recoveries		
			OD_{240}	BT*	Mean
A. Standards: 61 μg. corticosterone in 5 ml. ethanol (2)	0.576	0.375	—	—	—
B. Controls: 61 μg. corticosterone added to 80 ml. 40% methanol and extracted; residue dissolved in 5 ml. ethanol (2)	0.636	0.342			
Blanks† (2)	0.109	0.004			
Difference	0.527	0.338	91.6	90.2	90.9
C. Experimentals: 61 μg. corticosterone added to 10 ml. blood-water-methanol (1:1:1), dialysed into 80 ml. 40 per cent methanol and extracted; residue dissolved in 5 ml. ethanol (3)	0.834	0.406			
Blanks† (2)	0.324	0.066			
Difference	0.510	0.340	88.6	90.7	89.6

* Blue tetrazolium-choline test of Chen *et al.* (8).
† Blanks differed from controls or experimentals only by omission of corticosterone.

Extraction: The B controls in Table 1 give the value of 90.9% recovery. This figure was checked further by adding 202 μg. cortisone to 160 ml. of 40% methanol dialysate of peripheral blood taken from non-fasting rats, solvent extracting the dialysate, and determining steroid quantity as described above. Using triplicate samples and an appropriate blank, a recovery of 88.0% was obtained.

Transfer from Evaporating Dish: An ethanol solution of 63 μg. cortisone was added to 30 ml. methylene chloride standing in the evaporating dish illustrated in Figure 1. After evaporation to dryness, the steroid was transferred to a test tube with methylene chloride as already described. The methylene chloride was again permitted to evaporate and 5 ml. ethanol were added to dissolve the residue. The recovery obtained for triplicate samples, with correction for triplicate blanks, was 98.6%.

Chromatography: Table 2 gives the results obtained with chromatography and elution from the chromatogram. The B controls show that filter paper actually absorbs or adsorbs a significant portion of the steroid from a solution. Since D experimentals gave a recovery of 81.2% for the combined procedures of spotting, chromatography and elution,

TABLE 2. RECOVERIES OBTAINED WITH CHROMATOGRAPHY AND ELUTION FROM THE CHROMATOGRAM

Experiment (No. of determinations in parentheses)	OD at 240 mμ	OD for BT* test	Per cent recoveries		
			OD_{240}	BT*	Mean
A. Standards: 53 μg. cortisone in 5 ml. ethanol (3)	0.464	0.330	—	—	—
B. Controls: 4 cm. strip of clean filter paper shredded into solution of 53 μg. cortisone in 5 ml. ethanol (3)	0.477	0.317			
Blanks† (3)	0.039	0.008			
Difference	0.438	0.309	94.3	93.7	94.0
C. Controls: 53 μg. cortisone spotted on filter paper and 4 cm. strip eluted with 5 ml. ethanol (3)	0.410	0.288			
Blanks† (3)	0.039	0.008			
Difference	0.371	0.280	79.8	84.8	82.3
D. Experimentals: 53 μg. cortisone chromatographed on filter paper and 4 cm. strip eluted with 5 ml. ethanol (3)	0.515	0.276			
Blanks† (3)	0.136	0.009			
Difference	0.379	0.267	81.6	80.8	81.2

* Blue tetrazolium-choline test of Chen *et al.* (8)
† Blanks differed only by omission of cortisone.

C controls were run to obtain a recovery of 82.3% for spotting and elution. From these two values, the recovery for the chromatography procedure alone can be calculated as 98.6%.

Elution from the Chromatogram: C controls of Table 2 give recoveries of 82.3% obtained by mere elution of a 4 cm. strip of filter paper on which 53 μg. cortisone had been spotted. Experiments aimed at raising this recovery by means of multiple elution of the paper strips proved disappointing and lowered the reproducibility by increasing the risk of solvent evaporation during manipulation. Thus the above simple elution method was retained on account of its good reproducibility.

Entire Isolation Procedure: Table 3 summarizes the recoveries obtained in the five successive steps of the isolation procedure, giving a calculated recovery of 69.5% for the entire procedure. This compares with an actual recovery of 50.4%, obtained for the extraction of 61 μg. corticosterone from 10 ml. of a 1:1:1 mixture of peripheral blood (from fasting rats), water, and methanol, using triplicate samples and triplicate blanks. In a similar experiment in which 58.5 μg. corticosterone were isolated from pure 40% methanol, a recovery of 53.8% was obtained. This latter higher value is not unreasonable, con-

TABLE 3. SUMMARY OF RECOVERIES OBTAINED IN SUCCESSIVE STEPS OF THE ISOLATION PROCEDURE

Procedure	Per cent recovery
Dialysis	98.6
Solvent extraction	88.0
Transfer from evaporating dish	98.6
Chromatography	98.6
Elution from chromatogram	82.4
Calculated recovery for entire procedure	69.5

TABLE 4. R_f VALUES OF ADRENAL STEROIDS SECRETED BY NORMAL RATS
Chromatography at 37° in the Bush (7) system
Toluene-Methanol-Water (4:3:1)

Steroid	Known standard		Equivalent spot in extracts from rat adrenal vein blood	
	No. detns.	R_f	No. detns.	R_f
hydrocortisone (F)	2	0.24	—	—
cortisone (E)	7	0.37	—	—
component Y	—	—	14	0.62
corticosterone (B)	12	0.72	14	0.73
11-desoxy-17-hydroxycorticosterone (S)	4	0.72	—	—
11-dehydrocorticosterone (A)	2	0.78	2	0.78
11β-hydroxy-Δ^4-androstene-3,17-dione	2	0.84	13	0.86
Δ^4-androstene-3,17-dione	2	0.92	—	—

sidering the absence of impurities encountered in the methanol solutions that were obtained from blood eluates.

The present evidence suggests that the chromatographic recovery of steroids contained in blood extracts is considerably less than that of pure steroids. The best explanation may be that the chromatographic procedures produce chemical changes (for instance oxidation) in a certain percentage of the steroids present; these changes appear to be too minor to affect the optical density at 240 mμ or the reduction of BT, but apparently do affect the R_f value of the resulting modified steroid. Such a hypothesis would account for the appearance of a small amount of a UV-absorbing material of relatively high R_f value on chromatography of a steroid after it has been added to and extracted from peripheral blood.

In the present procedure, the BT test gave recoveries about 7% higher than those obtained using optical density at 240 mμ. This discrepancy may have been due to the lower specificity and higher blank values obtained using optical density at 240 mμ as a measure of steroid concentration. Nevertheless, the agreement between results was sufficiently close that the mean value given by the two methods was routinely used.

RESULTS FOR RAT ADRENAL VEIN BLOOD

Description of the cannulation procedure and quantitative data are given in the succeeding paper (18), where they permit comparison between the corticosteroid output of normal and pantothenic acid-deficient rats. The present paper gives qualitative data on the adrenal steroids secreted by normal rats during the first two hours of blood collection. Table 4 records the R_f value of these steroids, which were separated by the methods described above, and their identity is discussed below in order of increasing R_f value.

Hydrocortisone, Aldosterone and Cortisone: According to Bush (7) and Singer and Stack-Dunne (26), both hydrocortisone (compound F) and cortisone (compound E) are present in rat adrenal vein blood, though in unknown amounts and proportion. Whereas Singer and Stack-Dunne (26) have carefully determined the amount of aldosterone (with an R_f value intermediate between compounds F and E) secreted in rat adrenal vein blood, Rosenman *et al.* (24) have determined the total quantity of

17,21-dihydroxy-20-ketosteroids secreted, a figure that according to the present report includes compound E, F, and an incompletely identified steroid present in the chromatogram spot Y discussed below. In the present investigation, visual detection under UV light of compounds F, aldosterone and E was difficult or impossible on most strips. However, when the area corresponding to the R_f values of compounds F and E was eluted, the optical density of the eluate showed a peak at approximately 240 mμ. Both the OD_{240} and the BT tests were run routinely on the eluate, and the values obtained by the two tests were always higher than those for the corresponding blanks and permitted approximate quantification of the steroids.

Component Y: A dark band with R_f value 0.62 was found under UV in all chromatograms using the Bush (7) system and represents a steroid or steroids previously mentioned by Vogt (30). In the toluene-propylene glycol system of Zaffaroni and Burton (32, 34) the ratio of the rate of migration of the Y spot to that of corticosterone was approximately 0.53. Compound Y presumably was a Δ^4-3-ketosteroid, as evidenced by an absorption maximum at 241 mμ in ethanol solution. The sulfuric acid chromogens (33) of three different samples of Y all gave a single large maximum at 288–289 mμ. The phosphoric acid chromogen (22) of a sample of rechromatographed Y gave a single maximum at 285 mμ. On the basis of steroid concentrations calculated from the optical density at 240 mμ, assuming a typical extinction coefficient ($\epsilon_{240} = 15{,}800$) and molecular weight (346) for a corticosteroid, the Zimmerman reaction (29) with compound Y gave a light absorption at 520 mμ that was roughly 25% of that given by the same concentration of dehydroisoandrosterone. In contrast to 17-ketosteroids, the Zimmerman chromogen of Y gave a much stronger absorption at 350 mμ than at 520 mμ, the ratio of optical densities at 350 and 520 mμ being approximately 2.6. After elution from a single chromatograph, compound Y reduced blue tetrazolium (8) to give only about 12% of the light absorption at 515 mμ given by the same concentration of corticosterone; after rechromatography in the toluene-propylene glycol system, the reaction with the Silber-Porter reagent (25) was more vigorous and the wavelength-absorption curve for the chromogen gave the distinct maximum at 410 mμ that is characteristic of 17,21-dihydroxy-20-ketosteroids. These data seem to indicate that Reichstein's compound S is present in admixture with a nonreducing Δ^4-3-ketosteroid. The fact that neither the sulfuric nor the phosphoric acid chromogens of the two compounds agreed does not rule out the presence of S in small proportion. However, Table 4 records a 15% difference in R_f values which is significant and eliminates the possibility that S is a component of the Y spot.

Corticosterone: The corticosteroid present in highest concentration in rat adrenal vein blood is presumably corticosterone (compound B). Previ-

ous identification of this compound by Bush (7), Singer and Stack-Dunne (26) and Rosenman *et al.* (24) has been based on presumptive evidence such as R_f value, absorption in the UV and reducing properties. In the present investigation, this evidence was confirmed by agreement between the sulfuric acid chromogen of the rechromatographed spot from an extract of rat adrenal vein blood and the U.S.P. reference standard, as shown in Figure 2. Similarly, agreement between the phosphoric acid chromogen of

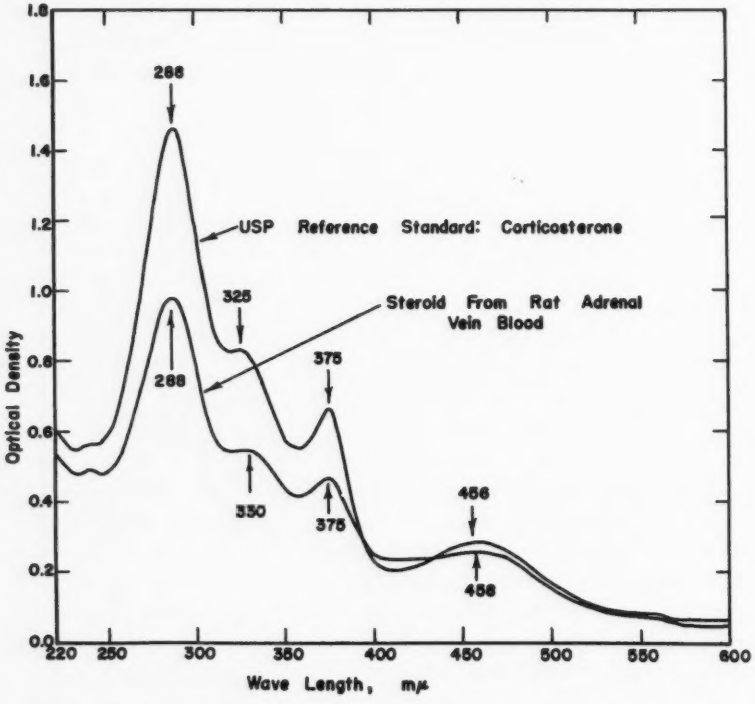

FIG. 2. Sulfuric acid chromogen of corticosterone and the corresponding steroid obtained from rat adrenal vein blood.

the unknown and standard steroids was good, with the unknown given maxima at 280, 377 and 460 mμ, while the standard gave maxima at 284, 378 and 460 mμ. The wavelength-absorption curve given by the unknown compound in the Silber-Porter reaction was identical with the curve given by the reference standard, and served to eliminate the possibility that compound S, which has an identical R_f value in the toluene-methanol-water system (Table 4), was present as a contaminant of corticosterone in any significant quantity. The data appear to constitute conclusive evidence of the presence of corticosterone in rat adrenal vein blood.

11-Dehydrocorticosterone: On two out of fourteen chromatograms of rat adrenal vein blood, a spot in the position corresponding to 11-dehydrocorticosterone (compound A) was seen under UV light. Bush (7) reported seeing this spot in the chromatogram of two out of eleven rats, while Singer and Stack-Dunne (26) reported traces of a component with an R_f value a little greater than corticosterone. In the present investigation, an eluate of this spot gave a sulfuric acid chromogen with a shoulder at 245 mμ, a major peak at 287 mμ and minor peaks at 355 and 415 mμ, in fair agreement with the chromogen for U.S.P. reference steroid A, that gave a major peak at 284 mμ and minor peaks at 354 and 416 mμ. These results are considered presumptive evidence in favor of the presence of compound A. The present interpretation is that the compound is present in the adrenal vein blood of rats, but in such small amounts that the chromatographic procedure is not sufficiently sensitive to permit visualization of the compound under UV on any but the sharpest chromatograms.

Component X: A UV-absorbing spot with an R_f value of 0.86 (Table 4) was consistently seen in all chromatograms of extracts of rat adrenal vein blood. Bush (7) labeled this spot compound X and indicated that it might be 11β-hydroxy-Δ^4-androstene-3,17-dione. Touchstone *et al.* (28) later isolated this steroid from incubates of human adrenals. In the present study, ethanol eluates of the X spot showed an absorption maximum at 240 mμ and consistently gave a Zimmerman reaction approximately one-half as strong as that given by the U.S.P. reference steroid 11β-hydroxy-Δ^4-androstene-3,17-dione, on the basis of solutions of equal concentration as determined by the absorption at 240 mμ. The Zimmerman chromogen of eluates of the X spot gave a ratio of approximately 2.4 for the absorption at 350 mμ to that at 520 mμ, while this ratio was 1.2 for the reference compound; these data indicate that the major steroid component of the X spot is probably not a 17-ketosteroid. While the reference compound gave a distinctive sulfuric acid chromogen, with major peaks at 283 and 380 mμ, a shoulder at 410 mμ and very minor peaks at 465 and 560 mμ, the sulfuric acid chromogen of eluates of spot X showed no major peak near 380 mμ. It is revealing that when an eluate of spot Y was rechromatographed, a UV-absorbing spot appeared in the X region, indicating that the X spot may conceivably contain a partially oxidized Δ^4-pregnene derivative. In the chromatography of other pure steroids, a very slight increase in the intensity of UV absorption in the X region was also occasionally noted, while no such increase in intensity was seen in control chromatograms. The present evidence suggests that if 11β-hydroxy-Δ^4-androstene-3,17-dione is a component of the X spot, it is contaminated with other Δ^4-3-ketosteroids of similar R_f value. These latter steroids may conceivably represent oxidation artifacts of the extraction and chromatography methods.

Since the above findings indicated the possibility of artifact formation,

a pooled sample of rat adrenal vein blood was extracted by a more conventional technique. Instead of alkalinizing the aqueous methanol extract with sodium carbonate, the methylene chloride extract was washed with one-eighth its volume of 0.2N sodium carbonate and then with one-eighth its volume of water and carried through the rest of the procedure. The appearance under UV light of chromatograms obtained using this method was identical with that observed with the method of choice.

Fluorescence: Fluorescence appeared near the solvent boundary of all chromatograms of extracts of rat adrenal vein blood, probably due to impurities. In addition, a fluorescent spot of R_f value 0.51 and unknown significance was seen on most of these chromatograms.

RESULTS FOR RABBIT ADRENAL VEIN BLOOD

Operative Technique: The operation for adrenal vein blood collection was similar to that used with rats (18), except for the following differences.[4] The left renal artery was ligated prior to insertion of a polyethylene cannula in the renal vein. After the other necessary ligations had been performed, adrenal vein blood was collected through this polyethylene cannula, which was 36 cm. long and of 1.3 mm. I.D. The cannula drained into a graduated tube placed in ice water about 12 cm. below the surface of the operating table, thus enabling a slight siphoning action to counteract the resistance to blood flow within the cannula.

Further differences from the experiments with rats were that Nembutal instead of ether was used as anesthetic. In a typical experiment with a 3.75 kg. rabbit, 2.5 ml. Nembutal (Abbott, 60 mg./ml.) was administered I.P. one hour prior to surgery, with 0.5 ml. given I.V. immediately before the start of surgery and 0.3, 0.15 and 0.10 ml. portions being given subsequently at one-hour intervals. Heparin (Hynson, Westcott and Dunning, 20 mg./ml. 0.85% saline) was given I.V. at the start of blood collection and at hourly intervals thereafter, the volume administered in a typical experiment being 0.9, 0.6, 0.4, 0.4 and 0.6 ml. Finally 5% glucose solution as prepared for human injection was slowly infused I.V. at the end of each hour's blood collection, the volume injected being 20% greater than the volume of blood collected in the previous hour, in order to partially compensate for blood volume lost through the cannula and through oozing from the site of the operation. During blood collection, the operative wound was covered with surgical cotton moistened with isotonic saline.

Adrenal Vein Blood Flow: Table 5 gives details of the six albino rabbits of mixed stock and sex that were used in these experiments. Each rabbit was treated identically, blood being collected hourly for three or four hours. In the fourth or fifth hour of blood collection, difficulties generally developed in maintaining a steady flow of blood through the cannula, and the experiment had to be terminated.

Table 5 shows that in every experiment the adrenal vein blood flow decreased over the four-hour collection period. The rate of blood flow in

[4] The help of Dr. T. L. Chiffelle in preparing an India ink-stained preparation of the venous system surrounding the left renal vein is gratefully acknowledged. This preparation was useful in demonstrating two veins (other than the adrenal vein) that drained into the relevant portion of the left renal vein. These two vessels were ligated in all operations.

TABLE 5. DESCRIPTION OF RABBITS AND RATE OF ADRENAL VEIN BLOOD FLOW
Blood from 2 rabbits was pooled in each experiment

Experiment	1	2	3	Mean
Sex of rabbits	M&F	M&F	M&M	—
Mean body weight, g.	2,865	3,820	2,975	3,220
Mean adrenal weight, mg.	77	244	144	155
Mean adrenal vein blood collected per rabbit, ml.				
1st hour	15.6	14.2	13.6	14.5
2nd hour	13.8	9.7	8.0	10.5
3rd hour	10.6	7.3	7.7	8.5
4th hour	6.8	—	7.2	7.0

the fourth hour was about one-half of the rate in the first hour of blood collection. The average amount of blood collected in the four rabbits that survived a four-hour collection period was 41.6 ml., or approximately 20% of their total blood volume.

Total Corticosteroid Output: Extracts of adrenal vein blood were prepared by dialysis and solvent extraction. These extracts were taken up in pure ethanol and analyzed by three spectrophotometric methods, without further chromatographic separation. Table 6 lists the results obtained for total Δ^4-3-ketosteroids, as determined by absorption of the ethanol solutions at 240 mμ. When these results are expressed on the basis of unit volume of adrenal vein blood, a surprising degree of constancy in hormone output over the four hours of the collection period is evident. Because the rate of blood flow decreased with time, the absolute rate of steroid output expressed on a time basis also decreases.

Production of Individual Steroids: Aliquots of the ethanol eluate were tested by the BT reaction (8) and by the Silber-Porter reaction (25). In experiments 1 and 3, respectively, 64.4 and 69.6% of the total Δ^4-3-ketosteroids as determined by UV absorption reacted in the BT test. In experiments 2 and 3, respectively, 8.4 and 10.9% of the total Δ^4-3-ketosteroids reacted with the Silber-Porter reagent. Neither the percentage of the BT-reacting steroids nor the percentage of the Silber-Porter-reacting steroids changed significantly during the four-hour collection period.

TABLE 6. TOTAL Δ^4-3-KETOSTEROID CONTENT OF RABBIT ADRENAL VEIN BLOOD
Results corrected for 10% loss during extraction procedure

Hour of blood collection	Expt. 1	Expt. 2	Expt. 3	Mean	Expt. 1	Expt. 2	Expt. 3	Mean
	μg. Δ^4-3-ketosteroids/ml. blood				μg. Δ^4-3-ketosteroids/adrenal/kg./hr.			
1st	9.5	26.0	24.9	20.1	57.1	95.2	72.3	74.9
2nd	14.4	25.8	31.1	23.8	69.1	64.9	83.1	72.4
3rd	15.1	29.7	25.9	23.6	56.5	60.8	66.7	61.3
4th	15.2	—	24.6	19.9	38.4	—	60.0	49.2
Mean	13.6	(27.2)	26.6	21.8	55.3	(73.6)	70.5	64.4

The four separate hourly samples from experiment 1 were chromatographed as described above and steroids with R_f values corresponding to hydrocortisone and corticosterone eluted and determined by absorption at 240 mμ as well as by the BT reaction. After correction for 50% loss during dialysis, extraction and chromatography, the mean output of the steroid moving with an R_f value comparable to hydrocortisone was 1.2 μg./adrenal/kg./hr., while the corresponding figure for corticosterone was 30 μg./adrenal/kg./hr.

DISCUSSION

The method for extracting unconjugated adrenal cortical hormones described in the present report consists of few steps, requires a minimum of specialized equipment and entails mild handling of the steroids. A recovery of 90% was obtained for dialysis and solvent extraction of 51 μg. corticosterone from 3.3 ml. fasting peripheral blood (Table 1). The recovery of individual steroids as eluates of chromatogram spots after dialysis and extraction from peripheral blood was 50%. The biggest loss in the present procedure occurred in chromatography and elution from the chromatogram. After trial of other elution methods, the described method was retained because of its simplicity and good reproducibility. The recoveries obtained with this method (Table 2) are in good agreement with comparable data of Bondy *et al.* (3). All recovery figures were obtained for conditions as similar as possible to those pertaining for extraction of adrenal vein blood, both with regard to the amounts of blood and of steroid present; the amounts of steroid used were relatively small compared to those used in testing recovery of similar methods (1, 17, 21). Finally, care was taken to subtract appropriate blank values from the recovery figures, and precautions were taken to avoid evaporation of solvent from the eluate of a steroid spot before final quantification. This latter error may easily occur unnoticed while transferring small volumes of alcoholic solutions, and results in high recovery figures.

The present method has been used to identify further the steroid hormones present in rat adrenal vein blood. Special attention had been given to component Y that had previously been noted by Vogt (30), which conceivably may be identical with a steroid of very similar chromatographic properties obtained by several workers on *in vitro* incubation of adrenal slices—compound X of Heard *et al.* (13), steroid II of Haynes *et al.* (12) and steroid 3 of Eisenstein (10). Heard *et al.* (13) found that in a mixed chromatogram of compound S and their compound X, there was demarcation between the C^{14} location of compound X and the added compound S; they concluded that spot C^{14}-X was probably not homogeneous, since on acetylation of its eluate, the paper radiochromatogram showed two well-separated photogenic areas. On the basis of indirect evidence, Eisenstein (10) surmised that at least a portion of his steroid 3 contained a 17,21-dihydroxy-20-ketone side chain. The present evidence suggests that com-

ponent Y of rat adrenal vein blood contains a steroid with the basic configuration of 17,21-dihydroxy-Δ^4-pregnene-3,20-dione (Reichstein's compound S), with some additional molecular modification. Comparing the apparent chemical constitution of this steroid with that of other known corticosteroids, the probability that this steroid may have physiological activity cannot be ignored.

As regards other steroids in rat adrenal vein blood, the presence but not the relative amounts of hydrocortisone and cortisone are indicated on presumptive evidence of this and previous investigations (7, 24, 26). The presence of aldosterone is indicated on the basis of the bioassay data of Singer and Stack-Dunne (26). The present investigation has given support to the presence of relatively small quantities of 11-dehydrocorticosterone. The precise constitution of Δ^4-3-ketosteroids moving with an R_f value greater than 11-dehydrocorticosterone is still in doubt. One single steroid has been shown to be a component of rat adrenal vein blood on more than presumptive evidence—namely corticosterone, the major corticosteroid constituent of rat adrenal vein blood.

The data obtained with rabbits do not show the initial depressing effect of Nembutal anesthesia, which might have been expected from the data that Beigelman *et al.* obtained with rats (2), very probably because the first Nembutal injection was given one hour before start of the operative procedure. The present result of an average total Δ^4-3-ketosteroid secretion of 64 μg./adrenal/kg./hr. are in agreement with the range of values of 31–73 μg./adrenal/kg./hr. which can be calculated from the data given by Bush (7). However, these results run 10- to 20-fold higher than those reported by Kass *et al.* (15). Thus, Kass *et al.* give an average figure of 1.6 μg. hydrocortisone plus corticosterone per ml. adrenal vein blood, compared to 14.6 μg./ml., which is calculated from the present results assuming that hydrocortisone and corticosterone are the only BT-reactive steroids and that they represent 67% of the total Δ^4-3-ketosteroid output. Kass *et al.* obtained an average blood flow of 4–6 ml. per hour, compared to 10.1 ml. per hour in the present report. In contrast to these discrepancies, which may be due to differences in rabbit stock or operational procedure, the ratio of the compounds which are presumably hydrocortisone and corticosterone agree well in all three investigations. Thus, the present ratio of approximately 0.04 compares well with "less than 0.10" obtained by Bush (7) and "less than 0.05" obtained by Kass *et al.* (15).

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SOY FLOUR AND FECAL THYROXINE LOSS IN RATS

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ABSTRACT

Rats fed soy flour and control diets were injected intravenously with tracer amounts of I^{131}-labelled thyroxine. Counts of radioactivity in collections of feces and in blood samples over a 90-hour period, and final whole carcass counts, confirmed the previous report of increased fecal excretion of thyroxine in soya-fed animals.

In investigating the mechanism of this effect rats fed on control and soy flour diets were subjected to biliary drainage over 12 hours through a polythene tube inserted into the common bile duct following intravenous injection of labelled thyroxine. The proportions of the injected radioactivity excreted in the bile, and present terminally in the gut, were measured. Samples of bile and butanol extracts of feces were chromatographed and the nature of the radioactive compounds determined. No differences were found between control and experimental groups in any of these parameters.

It was concluded that the excess thyroxine in the feces of soya-fed rats was not due to increased biliary secretion or to addition of thyroxine to the gut from any other source. Nor was any chemical alteration attributable to the soy flour demonstrated in biliary or gut thyroxine which would interfere with its reabsorption. The increased fecal thyroxine may therefore be due to some other upset of the absorption process, the result, perhaps, of accelerated transport or a specific effect of a soy flour factor on mucosal function.

A HIGH radioactive iodine uptake in simple goiter in the absence of iodine deficiency is a not infrequent finding. Drummy (1) recorded a 29% incidence of uptakes above the normal range in simple diffuse thyroid enlargement. The reasons for this phenomenon are not clear.

A parallel is found in the goiters of rats fed soy flour diets which have been shown by Astwood (2) to take up more tracer I^{131} per unit weight than control glands. Van Middlesworth (3) has demonstrated that the probable mechanism here is the fecal wastage of thyroxine which occurs in rats fed soy flour diets.

This new concept of a possible mechanism for goitrogenesis may well be of importance in relation to the problem of simple high-uptake goiters in human beings although the enterohepatic circulation of thyroxine is a much less prominent feature of the hormone's metabolism in the human than in the rat (4).

An attempt was therefore made to confirm and further to elucidate the results obtained by Van Middlesworth in rats.

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METHODS

In the investigation of fecal thyroxine excretion, twenty 100 to 140 gm. Sprague-Dawley rats were divided into two groups. For a preliminary three-day period control animals were fed a diet containing 20% casein, 36% sucrose, 30% starch, 8% corn oil, 5% mineral mixture and 1% vitamin mixture. The experimental group had 66% soy flour substituted for the starch and sucrose. Each animal then received 1.3 μg. of labelled thyroxine (23 μc) in 0.1 ml. 50% propylene glycol into the tail vein and was confined in a metabolism cage for fecal collections and tail blood sampling at intervals over a 90-hour period. The radioactivity of fecal and blood samples was measured using a scintillation counter. Counts were prolonged to give an error of less than 2%. Activity in feces was expressed as a percentage of an initial dose counted simultaneously with compara-

TABLE 1. DATA FROM BILIARY DRAINAGE EXPERIMENTS

Rat No.	Weight in grams	Preliminary diet		Dose		% of dose in 0–6 hr. bile	% of dose in 6–12 hr. bile
		Type	Duration in days	μc I^{131}	μg. thyroxine		
1	300	control	3	26	2.3	14.5	5.5
2	300	soya				14.3	5.6
3	325	control	4	100	8.5	17.7	8.8
4	300	soya				19.9	8.9
5	195	control	4	10	1.4	14.5	5.4
6	180	soya				14.2	6.0
7	270	control	5	100	8.6	9.5 (0–3 hrs.)	9.0 (3–12 hrs.)
8	290	soya				8.5 (0–3 hrs.)	8.0 (3–12 hrs.)
9	300	control	5	66	1.7	13.5	6.0
10	225	soya				17.8	6.2
11	200	control	5	10	1.6	13	5.5
12	180	soya				11.3	6.5
13	290	control	6	100	8.5	14.1	8.2
14	310	soya				16.9	8.5
15	190	control	7	10	0.6	13.2	6.8
16	190	soya				15.1	6.9
17	185	control	8	9.2	0.6	12.1	5.9
18	180	soya				15.4	6.7

ble geometry. When collections were completed the rats were killed and the whole carcass counted.

Measurements of biliary secretion of thyroxine were made in rats on control and soy flour diets. 180 to 325 gm. Sprague Dawley animals, 18 in all, were fed one or other diet for a period of 3 to 8 days. Bile collections were made through a fine polythene tube inserted into the common bile duct under ether anesthesia and were continued over 12 hours. Radioiodine-labeled thyroxine in 50% propylene glycol was given into the tail vein at the beginning of the collection period. For dosage see Table 1. Amounts of carrier thyroxine were within or little above the physiological range. Rats were operated on in pairs consisting of a control and an experimental animal and preliminary diet periods and thyroxine dosage were identical for each pair. Feces excreted during biliary drainage were also collected. The animals were then killed and the gut removed between the lower esophagus and the rectum. Radioactivity of 0 to 6- and 6 to 12-hour bile samples and of

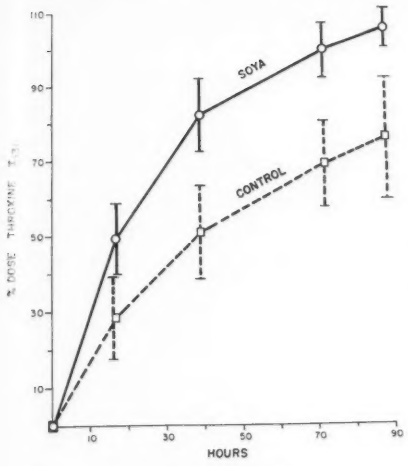

FIG. 1. Cumulative excretion rates of fecal thyroxine I^{131} with two standard deviations for each mean value.

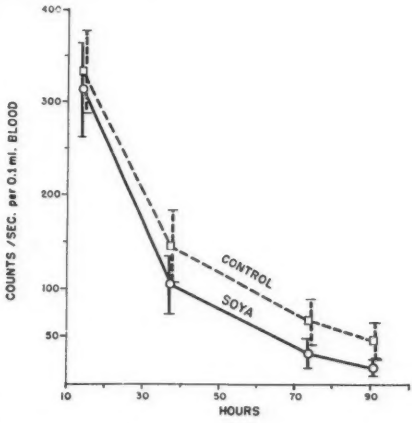

FIG. 2. Reduction in blood radioactivity in animals injected with thyroxine I^{131}; two standard deviatoins for each mean value.

gut plus feces was measured as before and compared with an initial dose counted in each case using similar geometry.

Samples of bile from both control and experimental groups were examined directly by ascending one-dimensional paper chromatography in butanol:water:glacial acetic acid, 75:15:10, and in butanol saturated with 2N ammonium hydroxide. The positions of radioactivity on the developed strips were detected by passing them slowly under a thin-window Geiger counter connected to an automatically recording rate meter.

To confirm the presence of thyroxine I^{131} glucuronides, bile samples were subjected to hydrolysis by incubation at 37° C for 12 hours at pH 5 with β-glucuronidase, in the presence of toluene as an antibacterial agent, and then rechromatographed. On several occasions, carrier thyroxine was added to the bile samples prior to chromatography and detected, after development, by the ceric ammonium sulphate-arsenious acid reaction.

The nature of the fecal radioactivity was investigated. Four pairs of 200 to 250 gm. Sprague-Dawley rats, fed for 4 days on control or soy flour diets, were injected intravenously with labelled thyroxine in 50% propylene glycol, the dose varying from 25 to 100 μc. I^{131} and 0.2 to 8 μg. thyroxine. Twelve-hour fecal collections were extracted twice with butanol. This removed approximately 70% of the radioactivity. After concentration samples of the butanol extract were chromatographed and the strips examined as before.

RESULTS AND DISCUSSION

The increase in fecal thyroxine excretion produced by soy flour diets and reported by Van Middlesworth (3) has been confirmed. A difference between control and experimental groups in the cumulative excretion figures for thyroxine I^{131} was found significant at the 95% confidence level, as early as 17 hours after the tracer dose (Fig. 1) changing to better than 99% at the later collection times. This effect is reflected in the more rapid decline of blood radioactivity in the soya-fed group which becomes statistically significant at the 95% confidence level 37 hours after administration of

the tracer and at the 99% level at 74 hours (Fig. 2). It is also consistent with the highly significant difference in residual carcass radioactivity between the two groups: 132 ± 36.1 c.p.s. for the control, 51.9 ± 19.5 c.p.s. for the soya group ($P > .001$).

This increased rate of elimination of thyroxine in the feces, induced by a soy flour diet, could be due to several mechanisms. Bearing in mind the substantial enterohepatic circulation of thyroxine in the rat (5), it is possible that (a) excessive quantities of thyroxine might be delivered to the gut by the liver; (b) the biliary thyroxine, although in normal amounts, might be secreted in a less readily absorbable form than the usual predominating glucuronides; (c) thyroxine might reach the gut in excess from a source other than the liver; (d) the soya diet might interfere with the hormone's reabsorption, by altering irreversibly its chemical nature, by increasing fecal bulk and producing "intestinal hurry," or by some more specific effect on the absorption process.

Tht results of the studies on biliary secretion of thyroxine I^{131} do not support the first hypothesis (Table 1). The means of the differences between control and experimental animals in the amounts of radioactivity secreted in the bile were small. For the 0–6 hour period, the value was 1.5% of the dose in favor of the soya group, and this was statistically significant only at the 90% confidence level. For the 6–12 hour collection, although significant at the 99% confidence level, the figure was relatively even less, 0.4%, again in favor of the soya-fed rats. It is improbable that these values have any biological significance when considered against the substantial differences in fecal thyroxine I^{131} excretion. For purposes of statistical evaluation, the animals were considered in pairs having comparable weights and the same preliminary diet periods and thyroxine I^{131} dosage. These biliary secretion values, although lower than those reported by Taurog (6) agree well with the results of VanArsdel and Williams (7) for normal rats.

The second possibility, the delivery of unusual thyroxine compounds in the bile, is excluded by the results of chromatography of bile samples from control and experimental animals. Identical patterns of distribution of radioactivity were obtained, namely into a major and minor peak, labelled G1 and G2 in Figure 3. Hydrolysis of bile with β-glucuronidase altered the position of most of the radioactivity to coincide with that of added carrier thyroxine—Figure 3. Both peaks seemed to undergo this change. It is probable that G1 is thyroxine glucuronide, as described by Taurog *et al.* (8) and Roche *et al.* (9). The identity of G2 is uncertain.

A source other than bile for fecal thyroxine, in the soya animals, could not be demonstrated. The radioactivity in the gut and feces of the rats with biliary fistulas, expressed as a percentage of dose, was small in amount, and the experimental and control group means and standard deviations for 5 pairs of rats were identical: 7.4 ± 1.6.

That absorption of the biliary thyroxine is not prevented by some irreversible chemical reaction with a factor in soy flour is suggested by the results of chromatography of butanol extracts of feces. The fecal radioactivity in all animals regardless of diet was shown to be in the form of free thyroxine.

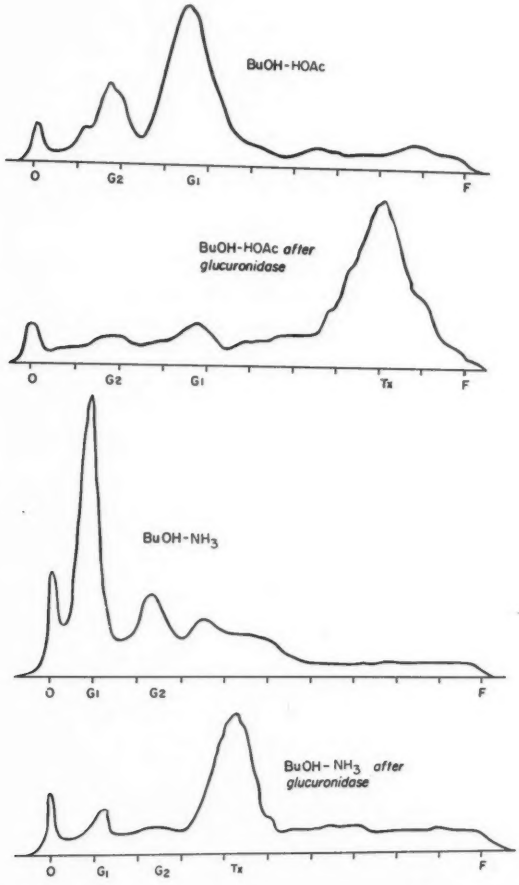

FIG. 3. Records of the radioactivity on chromatograms of bile in two solvent systems before and after hydrolysis with glucuronidase. Tx = thyroxine. O = origin. F = front. G 1 = thyroxine glucuronide. G 2 = identity uncertain.

Ham and Sandstedt (10) and Kunitz (11) have demonstrated the presence of a trypsin inhibitor in soy bean extracts. It has been pointed out by Almquist and Merritt (12) that amino acid deficiencies may be produced or accentuated by the addition of raw soy bean protein to the diet of chicks. Interference with proteolysis is not the complete explanation for

these results, however, as Westfall *et al.* (13) have shown that the soya "antitrypsin factor" also prevents absorption of already hydrolyzed protein. These findings may help to explain the excess excretion of thyroxine, itself an amino acid, when soy flour diets are fed.

It is therefore probable that the increase in fecal thyroxine content of rats on soya diets may be largely a function of incomplete reabsorption, due either simply to accelerated transport through the intestine or to some other more specific effect of the soy flour on mucosal function.

Acknowledgment

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EFFECT OF GROWTH HORMONE AND INSULIN ON BODY WEIGHT AND NITROGEN RETENTION IN PANCREATECTOMIZED RATS

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ABSTRACT

Female rats, weighing about 145 gm., were deprived of 95% of the pancreas and were tube-fed 7 gm. of food per day; pancreatin was not required. When given 12 units of insulin daily, the amount needed for the control of glycosuria (less than 0.1 gm./day), they gained 0.4 gm./day in body weight and retained 6% of the nitrogen fed. Administration of 0.1 mg. of growth hormone/day produced a body weight gain of 1.6 gm./day and retention of 28% of the nitrogen fed; 21% of the weight gain was protein. Extra food and extra insulin were not necessary for the growth hormone effect. Reduction of the dosage of insulin to 8 units/day impaired the growth response to growth hormone. This growth disturbance occurred before there was any significant increase in glycosuria or urine volume. When given 6 and then 4 units daily the rats treated with growth hormone continued to retain nitrogen but gained no weight. Although the growth hormone treated rats retained very little nitrogen when given 2 and 1 units daily, they retained significantly more nitrogen than did the pancreatectomized controls receiving the same amounts of insulin. Raising the insulin dosage from 1 to 4 units immediately abolished the nitrogen retaining effect of growth hormone. After increasing the insulin dosage to 12 units daily, which controlled glycosuria, both groups of rats gained body weight at a slow rate but neither retained nitrogen. There was no direct relation between the amount of nitrogen retained, with or without growth hormone, and the insulin dosage or glycosuria. When insulin was withheld two-thirds of the rats died in diabetic coma. Growth hormone (3.0 mg./day) had no effect on the insulin requirement of the diabetic rats.

IT HAS been demonstrated in several species that insulin is necessary for the anabolic action of growth hormone (1, 2, 3, 4). Studies in the cat and dog would suggest that increased secretion of insulin is needed for

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the maximal response to growth hormone (1, 2, 3). In hypophysectomized-pancreatectomized rats, however, growth hormone markedly stimulated growth without extra insulin (4). This species difference may be dependent on the fact that the rat, unlike the cat and dog (5), is relatively insensitive to the diabetogenic action of growth hormone (6). This paper presents additional studies of the relation between insulin and growth hormone in the rat.

EXPERIMENTAL

Female rats weighing about 145 gm. of the Instituto de Biologia y Medicina Experimental were pancreatectomized while under ether anesthesia. The operative procedure and the postoperative care were essentially the same as those described earlier (7). In the present study, however, only 95% of the pancreas was removed; the small lobules of pancreas along the bile duct were left intact. The average blood glucose level at 19 hours after pancreatectomy was 310 mg./100 ml. These rats needed daily 12 units of regular insulin[3] when fed 7 gm. of food/day to maintain glycosuria at less than 0.1 gm./day. Since the absorption of nitrogen and the appearance of the feces were normal, pancreatin was not given. (After extirpation of 99.5% of the pancreas, the fasting blood glucose 19 hours postoperative was 410 mg./100 ml., 18 units of insulin were needed with 7 gm. of food and pancreatin was necessary for normal absorption of food (7).)

The diet used, #4370, consisted of casein, 20%; L-cystine, 0.3%; dextrin, 66.7%; corn oil, 8%; salts, 4% and adequate amounts of all vitamins needed by the rat (7). An aqueous suspension of the diet, 0.5 gm./ml., was tube-fed twice daily with an interval of 6 or more hours between meals. (The operated rats were never permitted to eat ad libitum.) Regular insulin was injected subcutaneously prior to each feeding, 1–2 hours in the morning and less than 1/2 hour in the afternoon. The growth hormone,[4] which was highly purified (8), was dissolved in slightly alkaline 0.85% NaCl solution and was injected intraperitoneally twice daily.

The rats were kept in individual metabolic cages maintained at 26° to 28° C. They were permitted to drink water ad libitum. The procedures for collecting urine, feces, and food samples have been described (9). The nitrogen contents of the above were determined by the microkjeldahl method, glucose in urine by Benedict's quantitative method (10), glucose in the blood by the method of Haslewood and Strookman (11), and ketone bodies in the urine by the qualitative nitroprusside test (Rothera).

RESULTS

Experiment 1. This experiment was begun 3–7 weeks after removal of 95% of the pancreas (Fig. 1 and Table 1). Each rat was tube-fed 7 gm. of food daily throughout the experiment. Growth hormone was given only to one group of rats during the first 64 days of the experiment; thereafter the control group was also given growth hormone. Both groups of rats received the same amounts of insulin.

The pancreatectomized rats needed 12 units of insulin daily to maintain the urinary excretion of glucose at less than 0.1 gm./day. They gained 0.4

[3] Regular insulin was kindly supplied by Laboratorios Armour of Buenos Aires, Argentina.

[4] The purified growth hormone, obtained from beef pituitaries, was kindly supplied by Dr. C. H. Li of the University of California, Berkeley, California.

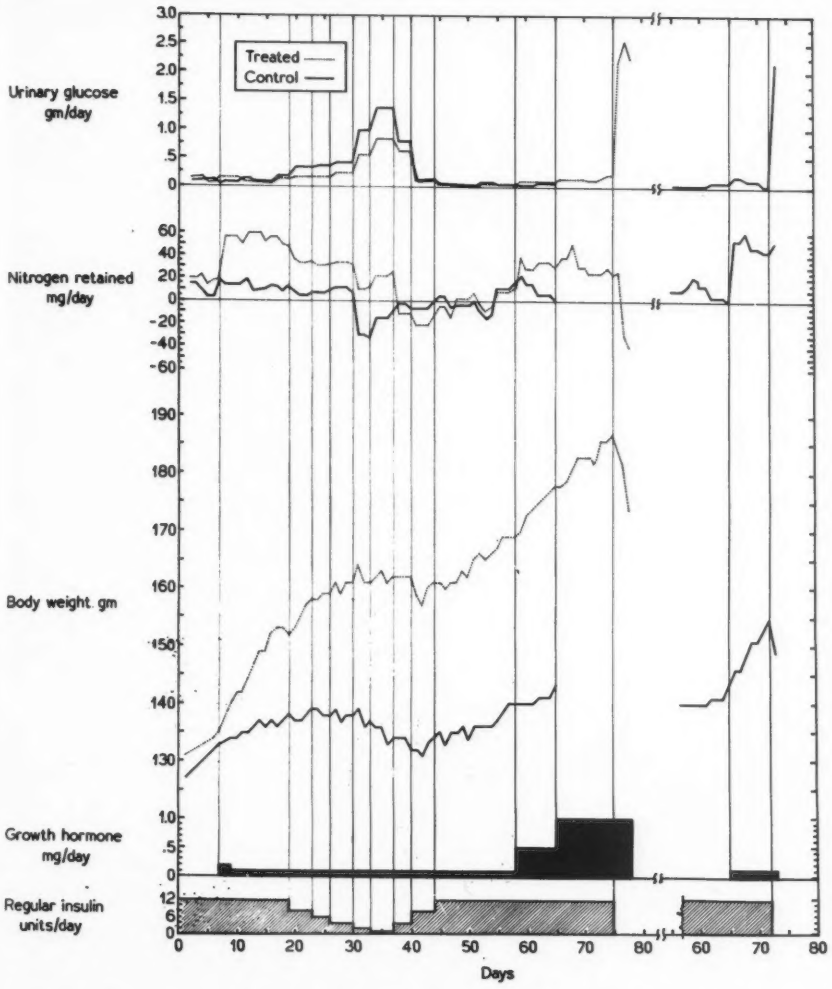

FIG. 1. *Experiment 1.* All rats were pancreatectomized 3–7 weeks before the experiment was begun. Each rat was tube-fed 7 gm. of food/day throughout the experiment. The Treated group was injected with growth hormone every day whereas the Control group was injected with growth hormone for only 7 days, starting on the 65th day. Both groups received the same amounts of insulin. There were 6 rats in each group until the 51st day when one growth hormone-treated rat was accidentally killed.

gm. of body weight per day and retained 6% of the nitrogen fed.[5] Administration of 0.1 mg. of growth hormone daily markedly increased the body weight gain and nitrogen retention of the pancreatectomized rats. Neither extra insulin nor extra food were needed for this effect. The ratio of nitro-

[5] The average nitrogen content of the diet was 28 mg./gm.

TABLE 1. EXPERIMENT I. EFFECT OF GROWTH HORMONE (GH) AND INSULIN ON NITROGEN RETENTION AND BODY WEIGHT IN PANCREATECTOMIZED RATS[a]

Insulin,[b] units/day	Growth hormone,[c] mg./day	Days of experiment	No. of days	Body weight gain, gm./day[d]		Nitrogen retained mg./day[d,e]		N retained × 6.25 / Weight gained × 100[d,f]		Urine volume, ml./day	
				GH-	Control	GH-	Control	GH-	Control	GH-	Control
12	0.1	7–19	12	1.6 ± 0.1	0.4 ± 0.1	53 ± 2*	12 ± 4	21.4 ± 0.9	25.3 ± 8.3	15	15
8	0.1	19–23	4	1.4 ± 0.5	0.3 ± 0.3	33 ± 5*	5 ± 5	14.0 ± 6.1	12.7 ± 10.6	16	16
6	0.1	23–26	3	0.7 ± 0.3	−0.3 ± 0.2	31 ± 8*	7 ± 3	45.7 ± 12.5	25.0 ± 18.1	15	18
4	0.1	26–30	4	0.3 ± 0.3	0.1 ± 0.3	33 ± 8	11 ± 8			16	18
2	0.1	30–33	3	−0.1 ± 0.3	−0.9 ± 0.3	11 ± 7*	−29 ± 10	2.5 ± 8.1	18.7 ± 7.5	18†	23†
1	0.1	33–37	4	0.3 ± 0.3	−0.5 ± 0.1	22 ± 10*	−12 ± 9			23†	28†
4	0.1	37–40	3	−0.6 ± 0.3	−0.5 ± 0.2	− 7 ± 12	−4 ± 4	33.4 ± 24.2	−6.3 ± 4.7	24	24†
8	0.1	40–44	4	−0.8 ± 0.5	0.5 ± 0.3	−19 ± 11	−6 ± 6			22	20
12	0.1	44–58	14	0.5 ± 0.1	0.4 ± 0.1	1 ± 3	1 ± 2	5.6 ± 4.7	−5.1 ± 7.5	20	20
12	0.5	58–65	7	1.2 ± 0.1	0.4 ± 0.1	32 ± 5*	11 ± 4	19.8 ± 3.4	25.6 ± 18.1	19	22
12	1.0	65–75	10	0.9 ± 0.3	—	31 ± 4	—	25.9 ± 5.9	—	22	—
12	0.1	65–72	7	—	1.7 ± 0.1	—	52 ± 5	—	19.3 ± 1.4	—	17†

[a] Only 95% of the pancreas was removed. The rats were tube-fed 7 gm. of food (192 mg. nitrogen)/day throughout the experiment. There were 6 rats in each group until the 51st day when one GH-treated rat was accidentally killed.

[b] Regular insulin was given to both groups.

[c] Growth hormone was given to the GH-treated group throughout the experiment whereas the control group was not injected with growth hormone until the 65th day.

[d] Mean ± standard error.

[e] The amount of nitrogen retained was determined as the difference between the amount fed and that excreted in the urine and feces. Throughout the experiment, in both groups, the nitrogen excreted in the feces, which were collected in 7 day periods, averaged 33 mg./day.

[f] If all retained nitrogen was deposited as protein, this ratio would give the per cent of the body weight gain that was protein.

* Statistically different from control ($P < 0.05$).

† Statistically different from previous period ($P < 0.05$).

gen retained to body weight gained would indicate that 21% of the body weight gain was protein (Table 1). (This is the same protein content as that in muscle (12).)

During the subsequent periods the daily dosage of insulin was reduced, stepwise, to 1 unit (Fig. 1). When 8 units were given daily (days 19–23), the pancreatectomized control rats no longer gained weight or retained nitrogen. The growth response to growth hormone was also affected by this small reduction in insulin dosage. During this period the growth hormone treated rats retained 40% less nitrogen than when given 12 units of insulin daily. Although some of the treated rats continued to gain weight, the average gain and the ratio of nitrogen retained to body weight gained were not significantly different from zero. There was no appreciable increase in glycosuria or urine volume associated with this growth disturbance.

The daily insulin dosage was reduced to 6 and then to 4 units during the next two periods (days 23–30). The rats treated with growth hormone continued to retain nitrogen although they gained no weight. The ratio of nitrogen retained to body weight gained was increased and was significantly different from zero, indicating a more uniform response to growth hormone than seen during the preceding period. The control rats gained neither nitrogen nor body weight during this time. There was no significant change in glycosuria or urine volume in either group.

When only 2, and then 1, units of insulin were given daily (days 30–37), glycosuria and urine volume[6] in both groups were increased ($P < 0.05$). The average urine volume, however, did not exceed 28 ml./day. Although the amount of glucose excreted daily was greater in the controls, the difference was statistically significant only when 1 unit of insulin was given daily. During these periods the control rats lost body weight and nitrogen whereas the rats treated with growth hormone maintained their body weight and nitrogen balance. The difference in nitrogen retention between the two groups, 34–40 mg./day, was significant ($P < 0.05$) (Table 1).

On the 37th day the insulin dosage was increased from 1 to 4 units and on the 40th day, to 8 units. In both groups of rats the glycosuria was reduced to less than 0.2 gm./day. The daily urine volumes were also reduced but were not as small as during the earlier period when 8 units of insulin were given daily (days 19–23). Furthermore, the nitrogen retaining effect of growth hormone disappeared instead of being enhanced when the insulin dosage was increased. The growth hormone treated rats no longer re-

[6] As the insulin dosage was decreased, the increase in urine volume in both groups was directly related to the amount of glucose excreted, 0.94 ml. for each 0.1 gm. of glucose. This would suggest that the rat kidney can not concentrate glucose in the urine any more than 10.6%. After increasing the insulin to 12 units/day, the daily urine volume was greater than during the initial period when the same amount of insulin was given (Table 1).

tained more nitrogen than the pancreatectomized controls. Increasing the insulin dosage from 1 to 8 units had little effect on the body weight or nitrogen retention of the controls.

Daily administration of 12 units of insulin was resumed on the 44th day. This dosage of insulin, even though given for 14 days, did not restore the response of the pancreatectomized rats to growth hormone. During this period both groups of rats gained 0.4–0.5 gm./day, but neither group retained nitrogen. When the dose of growth hormone was increased on the 58th day to 0.5 mg./day, the treated rats again retained nitrogen and gained weight at a faster rate. Increasing the growth hormone to 1.0

TABLE 2. *Experiment 1.* SURVIVAL, BODY WEIGHT LOSS, FINAL BLOOD GLUCOSE, KETONURIA, AND AMOUNT OF FAT IN LIVER AND KIDNEYS IN PANCREATECTOMIZED RATS AFTER STOPPING INSULIN[a]

Group	Survival time, days	No. of Rats	Body weight loss/rat, gm.[b]	Final blood glucose, mg./100 ml.[b]	Ketonuria	Fat in liver and kidneys[c]
Growth hormone-treated	3.5	2	19 ± 1	980 ± 25	yes	2–3
	8	3	13 ± 8	204 ± 53	no	0
Control	2	5	7 ± 6	1,020 ± 100	yes	2–3
	8	1	0	327	no	0

[a] These rats are described in Table 1 and Fig. 1. All rats had received 12 units of insulin daily for at least 3 weeks prior to the withdrawal of insulin. Tube-feeding of 7 gm. of food/day and the daily administration of growth hormone were continued after stopping insulin; the growth hormone-treated group received daily 1.0 mg. of growth hormone and the controls, 0.1 mg. The rats of each group were divided into 2 subgroups according to their response to insulin deprivation.

[b] Mean ± standard error.

[c] Amount of fat, graded from 0 to 3, observed in frozen sections stained by Wilson's oil red-O method. A detailed description of the fatty lesion in the kidney and liver has been presented elsewhere (17).

mg./day had no greater effect. The protein content of the body weight gain with the larger doses of growth hormone was 20–26% (Table 1).

Starting on the 65th day, the control rats were injected with growth hormone, 0.1 mg./day. They responded at once with a weight gain of 1.7 gm./day and a nitrogen retention of 52 mg./day. This growth continued as long as 12 units of insulin were given daily.

When the injections of insulin were stopped but administration of growth hormone and food was continued, two thirds of the rats died in coma (Table 2). These rats had terminal blood glucose levels around 1,000 mg./100 ml., ketonuria, fatty livers and fatty kidneys. The surviving rats, which showed no signs of coma and lived until killed on the 9th day, gained no weight and retained no nitrogen during this period.

The weights of various endocrine glands are given in Table 3. Administration of 20 mg. of growth hormone over a period of at least 10 weeks had no appreciable effect on the weight of these organs or on the histological ap-

pearance of the pancreas. Compared to unoperated rats tube-fed 7 gm. of food daily for 16 days, the pancreatectomized rats had small ovaries, uteri, and thymi, slightly enlarged adrenals, and normal sized pituitary and thyroid glands. The weight of the remaining pancreas, located along the bile duct, was about 5% of that of the organ at the time of pancreatectomy. Histological study of the pancreatic remnants demonstrated that the acinar cells were normal, the islets of Langerhans were small and few in number, and the proportion of islet to acinar tissue was greatly subnormal. There were beta cells, but no alpha cells, in the islets. Since many of the pancreatectomized rats died in coma (see Table 2), it is possible that some

TABLE 3. *Experiment 1.* WEIGHTS OF VARIOUS ENDOCRINE ORGANS AND UTERUS IN PANCREATECTOMIZED AND NORMAL RATS[a]

Group[b]	No. of rats	Body weight, gm.		Pancreas (mg.)	Pituitary (mg.)	Thyroid (mg.)	Adrenals (mg.)	Ovaries (mg.)	Uterus (mg.)	Thymus (mg.)
		Maximum	Final							
Pancreatectomized Growth hormone-treated	6	183±7	166±6	21.7±3.3	6.5±0.5	10.3±1.7	45.1±1.5	21.1±1.2	150±12	60±16
Control	6	155±4	144±7	18.3±3.1	7.0±0.5	11.2±1.2	41.7±0.7	19.1±1.7	128± 9	67± 4
Normal	6	—	148±5	(425)[c]	7.6±0.4	12.2±0.5	37.6±1.6	34.3±2.2	251±32	265±13

[a] Mean ± standard error.
[b] The pancreactectomized rats were autopsied at end of the experiment described in Table 2. The growth hormone-treated group had received a total of 20 mg. of growth hormone/rat; the pancreatectomized controls, 0.7 mg./rat. For 16 days prior to autopsy the normal rats were also tube-fed 7 gm. of food daily; their average body weight gain was only 3 gm.
[c] Average weight of the pancreas taken from 8 normal female rats, fed ad libitum, weighing about 145 gm.

of these organ weights are not the same as those in rats maintained on insulin.

Experiment 2. The purpose of this experiment was to determine the effects of different amounts of growth hormone in pancreatectomized rats given daily 7 gm. of food and 12 units of insulin. Daily administration of 0.01 and 0.03 mg. of growth hormone had no significant effect on nitrogen retention (Fig. 2). Both the rate of body weight gain and nitrogen retention were increased when 0.1 mg. was given daily.[7] With larger amounts, up to 3 mg./day, there was no additional increase in nitrogen retention and body weight gain. There was an insignificant increase in glycosuria with 1 mg. growth hormone/day but not with 3 mg. When growth hormone injections were stopped, all rats immediately went into negative nitrogen balance and lost 10 gm. of body weight in 6 days.

[7] In Experiment 2 (Fig. 2), the ratio

$$\frac{\text{nitrogen retained} \times 6.25}{\text{body weight gained}}$$

was 10.6±7.5% when 0.01 to 0.03 mg. of growth hormone were given daily and 22.2 ±1.1% when 0.1 to 3.0 gm. were given. The low ratio, 11.3±1.0%, seen during the period of rapid body weight gain when 0.1 gm. of growth hormone was given daily is probably due to the large water gain typical of the initial response to growth hormone (21).

DISCUSSION

These results confirm the earlier finding (4) that the rat, unlike the dog and cat (1, 2, 3), does not need extra insulin for the anabolic action of growth hormone. This species difference is probably related to the relative insensitivity of the rat to the diabetogenic action of growth hormone (6).

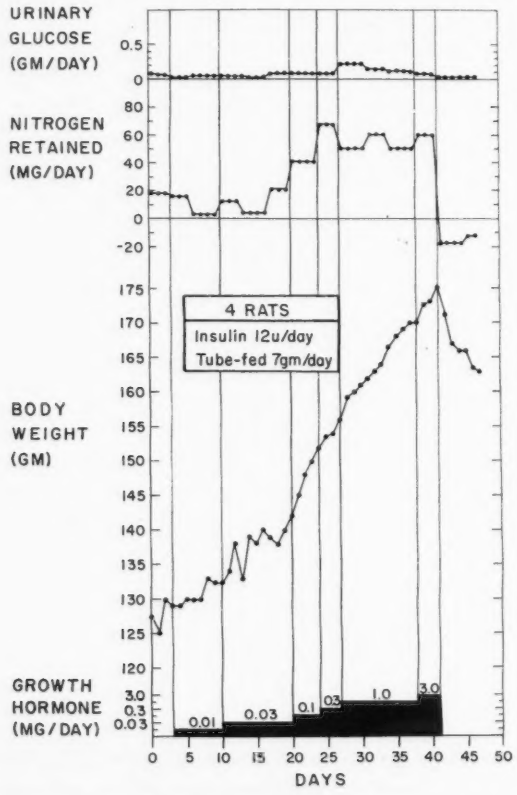

FIG. 2. *Experiment 2.* This experiment was begun 3 weeks after pancreatectomy.

The latter is supported by the observation that the administration of large amounts of growth hormone, 1 mg. daily for 11 days and 3 mg. daily for 3 days, has no effect on the insulin requirement of the pancreatectomized rat (Fig. 2).

Reduction in insulin dosage impaired the growth response to growth hormone before there was any increase in glycosuria and urine volume (Table 1, Fig. 1). It was also observed that the effect of the insulin deficiency on the growth hormone response was not readily corrected by the administration of insulin. These observations would strongly indicate that

the role of insulin in body growth is more complicated than just the enhancement of glucose uptake (4, 13).

It is possible that insulin failed to restore the growth response to growth hormone in the pancreatectomized rats because of the development of a resistance to growth hormone unrelated to diabetes. As discussed below, the pancreatectomized rats had a marked sensitivity to growth hormone, similar to that in hypophysectomized rats (4, 9, 14, 15). Simpon *et al.* (14) observed a steady weight gain for 120 days in hypophysectomized rats given the same dosage of growth hormone as that used in this study. If the responsiveness of the pancreatectomized rats to growth hormone was not affected by the insulin deficiency, one would expect them to continue to respond to growth hormone for longer than 37 days. On the other hand the observation that nitrogen retention was resumed when the growth hormone dosage was increased, 14 days after increasing insulin to 12 units/day, would support the idea that resistance to growth hormone had developed. However, this may have occurred without the extra growth hormone, in view of the nitrogen retention (11 ± 3 mg/day) during the 4 days just before the growth hormone dosage was increased (Fig. 1). It is not known whether the large dosage of growth hormone would have elicited a growth response if given earlier, or whether the control rats would have responded to the small dosage of growth hormone if it had been started earlier. These points require further study.

Body weight gain and nitrogen retention are the two most commonly used measurements of growth in animal studies. If all of the nitrogen retained is converted to protein, the protein content of the body weight gain can be estimated by the ratio,

$$\frac{\text{nitrogen retained} \times 6.25}{\text{body weight gained}}.$$

It is generally safe to conclude that body growth has occurred if the value of this ratio is the same as that for the protein content of the whole carcass (20% in the rat (12)) and the weight gain is significant. Such was the case in the pancreatectomized rats when given growth hormone and 12 units of insulin daily during the first (days 7–19) and last (days 58–75) parts of Experiment 1 (Table 1). When the value of this ratio differs from that for the protein content of the carcass, interpretation of nitrogen balance studies becomes difficult. This ratio for the growth hormone treated rats was reduced to 14 when the insulin was decreased to 8 units/day. Since all of the treated rats retained nitrogen, the decreased value for this ratio and its large standard error reflect the fact that the weight gain of some of the rats was markedly reduced. When 6 and 4 units of insulin were given daily this ratio had a value of 45.7%, suggesting that the nitrogen retained during this period was used for forming tissue with a low water content, such

as hair, skin and bone. The nitrogen and weight gains, as well as the ratio, were essentially zero in the treated rats when the insulin dosage was reduced to 1 unit and then raised to 8 units/day. When 12 units of insulin were given daily (days 44–58), the growth hormone treated rats gained body weight but did not retain nitrogen. The very small value for the ratio for this period would suggest that this weight gain was either the result of fat synthesis or the retention of water, or both. A similar gain was seen in the untreated rats.

Another possible explanation for the retention of nitrogen without weight gain seen in the growth hormone treated rats as the insulin was decreased (Table 1) is that all types of tissues were being formed, instead of only those with a low water content, but the expected body weight gain was masked by the loss of water with glucose in the urine as the insulin dosage was reduced.[6] The weight gain observed later when 12 units of insulin were given daily would then be the result of rehydration, which unmasked the tissue formed earlier. This hypothesis receives some support from the fact that the ratio

$$\frac{\text{nitrogen retained} \times 6.25}{\text{body weight gained}}$$

for these periods combined, days 19–58, had a value of 16.4 ± 2.4. The chief objection to this hypothesis is that the growth response to growth hormone was impaired before there was any significant increase in glycosuria and urine volume (Fig. 1 and Table 1). Another objection is that it seems unreasonable that such a long time, at least 14 days, would be required for rehydration after reducing glycosuria to the initial low level. It is possible that the nitrogen retained was initially stored as non-protein substances and at a later time, as the rats were gaining weight, it was used for tissue formation. A possible "site" for the temporary storage of nitrogen could be the stomach and intestines. The amount of nitrogen involved, 395 mg., is equivalent to that fed in two days. Since nitrogen retention did not occur in the controls, which were given the same amounts of insulin and food, storage of nitrogen in the gut is unlikely unless one proposes that growth hormone impairs absorption of nitrogen from the gut in the poorly controlled diabetic.

The finding that only 0.1 mg. of growth hormone daily was necessary for growth in the pancreatectomized rats when given 12 units of insulin/day would suggest that they were deficient in growth hormone (4, 9, 14, 15). The small size of the ovaries and uteri would indicate that the pituitary secretion of gonadotrophic hormones was also impaired (Table 3). On the other hand, the large adrenal glands and the development of diabetic coma when insulin was withheld (Table 2) demonstrates that the secretion of adrenocorticotrophic hormone was not impaired (16). Although some of

the changes in pituitary function (e.g., secretion of gonadotrophic hormones) could be an effect of pancreatectomy which insulin could not correct, the most likely explanation for the growth hormone deficiency is that the food intake, adequate for the response to exogenous growth hormone, was insufficient to maintain normal pituitary function. The inadequacy of the food intake is shown by the fact that in the present study normal female rats gained less than 3 gm. in 16 days when tube-fed 7 gm. of food/day (Table 3).

When insulin was withheld two-thirds of the pancreatectomized rats died within 2 days in coma whereas the other third lived 8 days without developing ketonuria, fatty livers and fatty kidneys. Recent studies have shown that whether diabetic rats develop coma when deprived of insulin depends largely upon their body fat contents (17). The difference in response to insulin withdrawal is probably due to a difference in fat content of the carcass. Chronic administration of growth hormone, instead of increasing the incidence and severity of coma as seen in the dog (18), may have given some protection to the rats (Table 3) by decreasing the amount of fat in the body (19). Growth hormone had no detectable effect on the pancreatic fragments (20).

Acknowledgments

The authors are grateful to Dr. Bernardo A. Houssay for his stimulating guidance and to all members of the Instituto de Biologia y Medicina Experimental for their cordial hospitality. The authors also express their gratitude to Dr. Aldolfo Cardeza for the histological study and to John Bowkett and Marcelino Cereijido for their excellent assistance.

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THE EFFECT OF A LIPIDE EXTRACT FROM THE POSTERIOR HYPOTHALAMUS AND OF PITRESSIN ON THE RELEASE OF ACTH FROM THE PITUITARY GLAND[1]

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ABSTRACT

The effect of a nonsaponifiable lipide extract of bovine posterior hypothalamus and of Pitressin on the corticotrophin secretion from the hypophysis was measured on the adrenal ascorbic acid content under different experimental conditions.

In normal and in nembutalized rats both substances caused an ascorbic acid depletion. This effect was abolished in hypophysectomized rats.

Pharmacological blockade with morphine or hydrocortisone inhibited the effect of the lipide extract. Only morphine was able to prevent the adrenal activation by Pitressin.

Electrolytic lesions destroying the median eminence, but not the hypophysial stalk, abolished the action of the lipide extract, whereas Pitressin was still highly active.

It is concluded that the lipide extract requires the intactness of hypothalamic structures for its action, and that Pitressin contains the specific neurotransmitter.

IN 1948 Green and Harris (1) postulated that the release of adrenocorticotropic hormone (ACTH) from the adenohypophysis is controlled by a hypothalamic neurohumor.

Several attempts have been made to isolate this principle (2, 3, 4). As is generally known various substances are able to cause ACTH discharge from the hypophysis. These factors presumably do not influence the adenohypophysis directly, but they may act somewhere on the brain stem resulting in the elaboration of the corticotrophin-releasing factor (CRF) from diencephalic centra. Therefore this substance must be able to stimulate the pituitary directly, i.e., independent of hypothalamic influence. Several experiments indicate the existence of a humoral transmitter. Porter and Jones (5) demonstrated ACTH-releasing activity of hypophysial portal vessel blood in hydrocortisone-treated rats, Shapiro *et al.* (6) found the same in blood obtained from electro-shocked rats in hypothalamic-lesioned animals, whereas Guillemin and Rosenberg (7) have shown a similar activity in hypothalamic extracts on surviving pituitary tissue.

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Slusher and Roberts (8) prepared a nonsaponifiable lipide extract of the posterior part of the hypothalamus which stimulated the release of ACTH in normal rats as measured by adrenal ascorbic acid depletion.

Another substance was isolated by Saffran *et al.* (9). These authors have shown that a polypeptide contaminating Pitressin induced a release of ACTH from surviving hypophysial tissue.

The present paper deals with the effect of the lipide extract and of Pitressin on pituitary function under various experimental conditions in order to investigate their possible independence of the hypothalamus in their ACTH-releasing capacity.

MATERIAL AND METHODS

In all experiments adult albino rats of an inbred strain, kept at an environmental temperature of 22° C and fed *ad libitum* on a standard diet, were used.

A nonsaponifiable lipide extract was prepared according to the procedure described by Slusher and Roberts (8) from fresh bovine posterior hypothalamus. Of this extract 10 mg. was suspended in saline just before administration. It was injected intraperitoneally in a volume of 0.2 ml. per 100 gm. body weight.

Pitressin (Parke, Davis) was injected intravenously into the tail vein in an amount of 0.2 ml. per 100 gm. body weight containing 0.2 I.U. As a control solution the same amount of saline was administered in the same way.

Pituitary function was tested by measuring the adrenal ascorbic acid content. One and a half hours after treatment the rats were sacrificed by decapitation. Both adrenal glands were removed, dissected clean and weighed. Ascorbic acid determination was carried out according to Roe and Kuether (10) as modified by Sayers *et al.* (11).

The effect of both substances was determined under the following conditions:

A. *Experiments with normal and hypophysectomized rats*

In the experiments described under A and B, male rats weighing 140–190 gm. were used.

In these experiments the normal rats were accustomed to treatment by injecting 0.5 ml. saline intravenously and 0.5 ml. intraperitoneally during three consecutive days, starting the 4th day preceding the experiment.

For the experiment with hypophysectomized animals, the pituitary gland was removed 22–24 hours previously by the parapharyngeal approach. Gross examination of the sella was performed at autopsy and data from animals with hypophysial remnants were discarded.

B. *Experiments in rats subjected to pharmacological blockade*

The following blocking agents were used:

Nembutal (Sodiumpentobarbital). This drug was administered intravenously in a dose of 3.25 mg. per 100 gm. body weight. The animals were adapted to treatment by injecting 3 mg. of the barbiturate on the 4th, 3rd and 2nd day before the experiment.

Nembutal+Morphine. In this case per 100 gm. body weight 3 mg. Nembutal was injected intravenously; after 20 minutes 2 mg. morphine hydrochloride was administered via the intraperitoneal route, and another 20 minutes later the substances under investigation were injected. The rats used in this experiment were adapted to treatment as in the preceding experiment.

Hydrocortisone. Hydrocortisone acetate was injected intraperitoneally in a dose of 5 mg. per 100 gm. body weight 4 hours prior to the actual experiment.

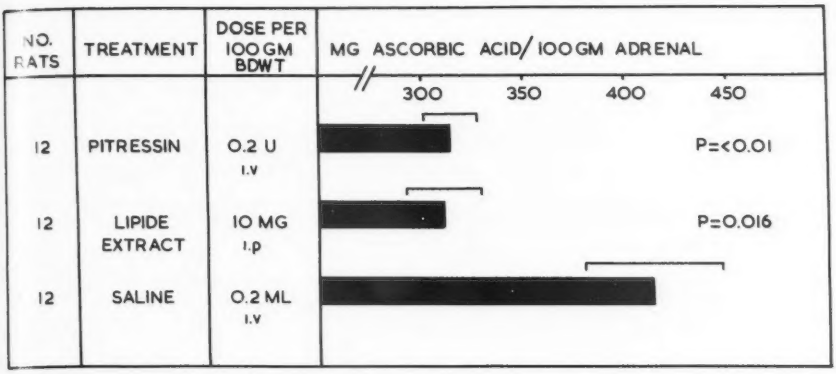

FIG. 1. The effect of Pitressin and of a nonsaponifiable lipide extract of bovine posterior hypothalamus on the adrenal ascorbic acid response of normal male rats. (The brackets represent the standard error of the mean.)

C. *Experiments in rats bearing hypothalamic lesions*

Female albino rats weighing from 160–180 gm. of the same inbred strain were used. Lesions were produced by means of a modified Krieg stereotaxic instrument as described by Greer *et al.* (12). A detailed description of the experimental procedure is published elsewhere (13).

Extensive lesions were placed in the basal hypothalamus destroying the whole median eminence and the rostrally adjacent regions. The hypophysis and the hypophysial stalk remained intact.

On the 3rd day following the operation Pitressin, lipide extract or saline were injected. The left adrenal gland was removed under ether anesthesia by a lateral skin incision 1½ hours later and the ascorbic acid content was determined. The right adrenal gland was removed after 7 days.

Sham-operated animals were submitted to the same experimental procedure and provided control data on compensatory hypertrophy.

In normal rats after unilateral adrenalectomy a compensatory hypertrophy of the remaining adrenal gland occurs. Assuming that the inability of the adenohypophysis to maintain the normal rate of ACTH production results in an inhibition of this compensatory hypertrophy, the absence of the hypertrophy was used as a criterion for the effectiveness of the hypothalamic lesion.

Compensatory adrenal hypertrophy was calculated as percentage of the weight of the left adrenal gland.

The location and extent of the lesions were examined in serial sections through the hypothalamus after staining with a modified azan method.

The statistical analysis of the results was performed using Wilcoxon's two sample test (14, 15). A difference was considered as statistically significant, if the double tail probability ≤ 0.05.

RESULTS

Ad A. As is shown in Figure 1 the adrenal ascorbic acid content of saline-treated normal rats treated with Pitressin amounted to 314 ± 10.6 mg.%. Treatment with Pitressin or lipide extract appeared to cause an equal as-

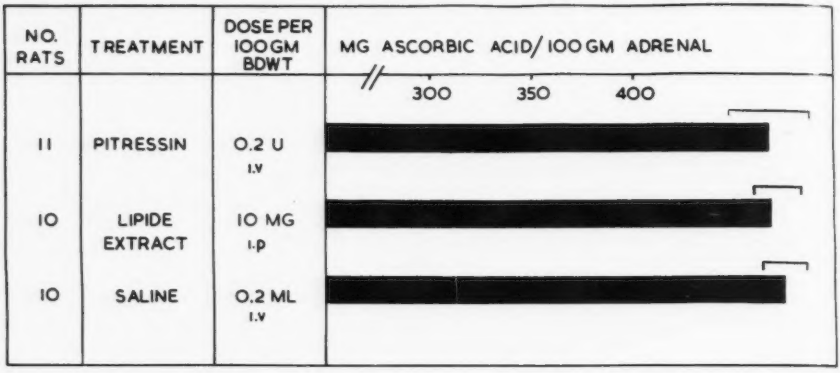

FIG. 2. The effect of Pitressin and of a nonsaponifiable lipide extract of bovine posterior hypothalamus on the adrenal ascorbic acid response of hypophysectomized male rats. (The brackets represent the standard error of the mean.)

corbic acid depletion, the ascorbic acid content being 314 ± 10.6 mg.% and 312 ± 10.6 mg.% respectively. Hence in normal rats at the dosage levels used, the two compounds exerted a similar effect on the ACTH secretion.

In hypophysectomized rats (Fig. 2), neither Pitressin nor the lipide extract were able to affect the ascorbic acid level, which indicates that the effect requires the presence of the pituitary gland.

Ad B. If Nembutal was used as a central depressant, Pitressin caused a somewhat smaller ascorbic acid decrease than in normal rats as is seen from Figure 3, although the difference between treated and control rats was statistically significant ($P = 0.02$). Under these conditions the effect of the lipide extract was similar to that in nonanesthetized rats.

Figure 4 shows that nembutalized rats treated with morphine did not re-

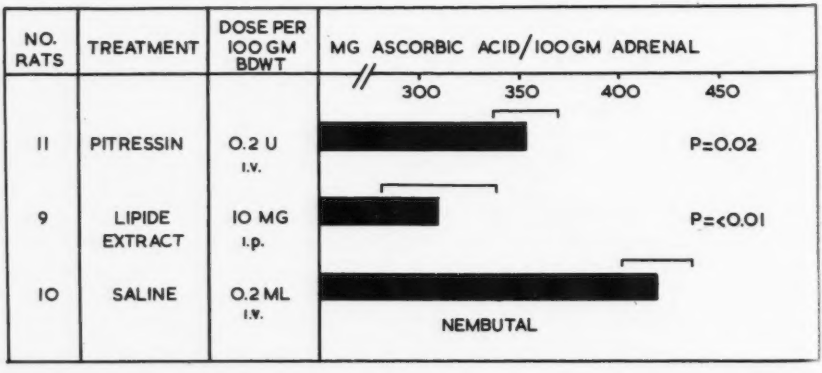

FIG. 3. The effect of Pitressin and of a nonsaponifiable lipide extract of bovine posterior hypothalamus on the adrenal ascorbic acid response of nembutalized male rats. (The brackets represent the standard error of the mean.)

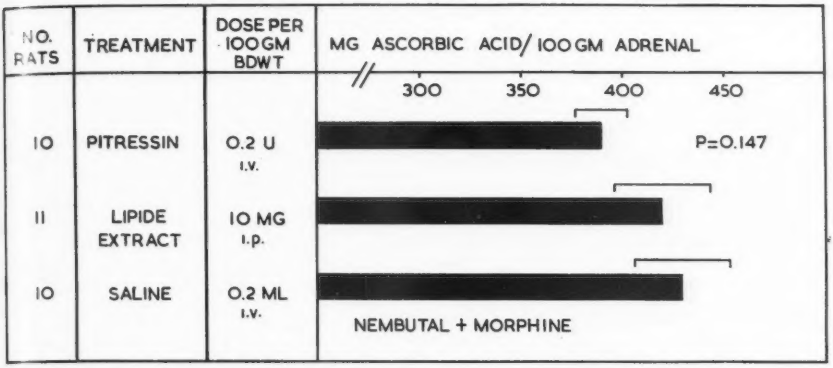

FIG. 4. The effect of Pitressin and of a nonsaponifiable lipide extract of bovine posterior hypothalamus on the adrenal ascorbic acid response of morphine-treated nembutalized male rats. (The brackets represent the standard error of the mean.)

spond materially to any of the injected compounds. The ascorbic acid content in rats treated with Pitressin amounted to 392 ± 9.7 mg.%; that of rats treated with the lipide extract being 421 ± 18.7 mg.%, while control values amounted to 424 ± 18.8 mg.%.

In the experiment in which hydrocortisone was used as a blocking agent Pitressin induced a significant ascorbic acid decrease, whereas the activity of the lipide extract was completely prevented (Fig. 5).

Ad C. The results from the experiment with rats bearing hypothalamic lesions in the median eminence are summarized in Figure 6. The adrenal ascorbic acid level in Pitressin-treated rats differed significantly from that in saline-treated animals ($P < 0.01$). The lipide extract was not able to cause a significant ascorbic acid decrease in the lesioned rats.

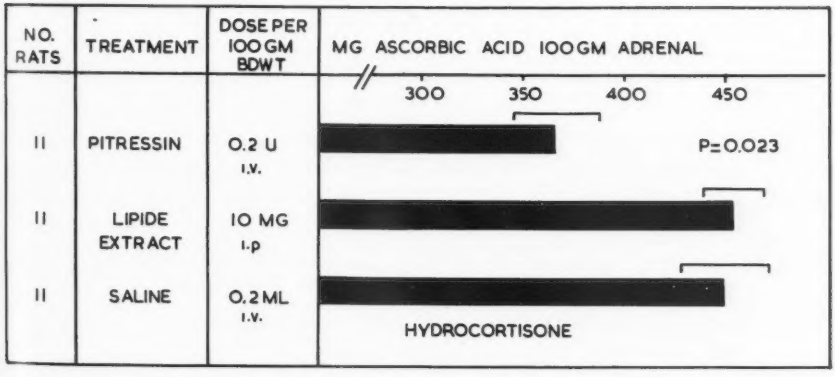

FIG. 5. The effect of Pitressin and of a nonsaponifiable lipide extract of bovine posterior hypothalamus on the adrenal ascorbic acid response of hydrocortisone-treated male rats. (The brackets represent the standard error of the mean.)

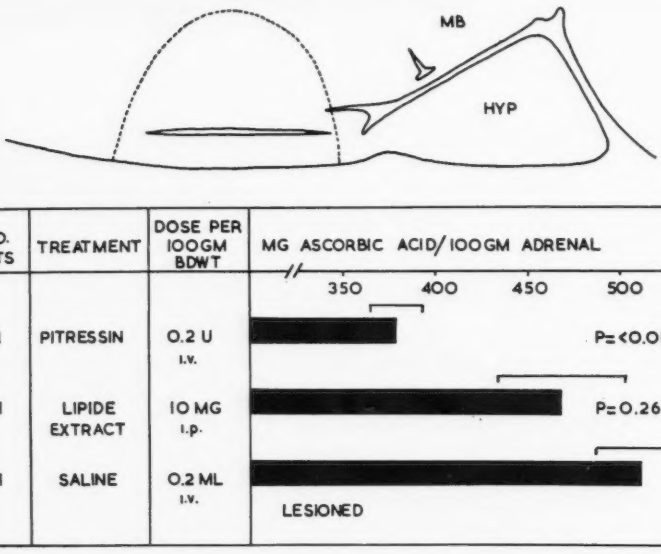

FIG. 6. The effect of Pitressin and of a nonsaponifiable lipide extract of bovine posterior hypothalamus on the adrenal ascorbic acid response of female rats bearing hypothalamic lesions. (The brackets represent the standard error of the mean.)

The effectiveness of the lesion was judged by the occurrence of compensatory hypertrophy of the remaining right adrenal. In none of the lesioned animals compensatory hypertrophy occurred, as may be seen from Table 1. Histological examination of the hypothalamus revealed that in all lesioned animals the destroyed area was nearly the same.

DISCUSSION

The present results show that a nonsaponifiable lipide extract of bovine posterior hypothalamus is able to decrease the adrenal ascorbic acid content in normal rats. This effect was not observed in hypophysectomized animals, which indicates that the extract requires the presence of the pituitary gland for its activity. These results confirm the earlier findings of Slusher and Roberts (8).

TABLE 1. MEAN WEIGHT OF ADRENAL GLANDS, SHOWING CHANGE IN WEIGHT DURING 7 DAYS FOLLOWING UNILATERAL ADRENALECTOMY

Procedure	No. of rats	Mean weight/100 gm. body wt.		Difference in %
		Left adrenal	Right adrenal	
Lesioned	34	17.6 ± 0.4*	13.5 ± 0.4	−23.4
Control	30	14.8 ± 0.4	17.4 ± 0.8	+15.2

* Standard error of the mean.
The figures differ highly significant from each other in horizontal and vertical direction.

Pitressin possesses the same properties as has already been shown by several other investigators (16, 17).

It is generally known that many compounds cause a release of ACTH from the pituitary. Treatment with Nembutal abolishes the stimulating effect of some of these compounds, e.g., of sodium salicylate, pyramidone and sulfamerazine (17), morphine (18, 19), chlorpromazine (20) and atropine (21). A limited number of substances, however, are still able to stimulate the adenohypophysis after injection of Nembutal. Among them are histamine (18, 20), epinephrine (17, 18, 19, 20, 22), norepinephrine (20), serotonin (19) and carbachol (23). These substances have in common that they naturally occur in the organism and for this reason it may be possible that their ACTH-releasing capacity represents a more specific functional pattern. Both the lipide extract and Pitressin can be considered as belonging to this group of substances, for they also retain their activity in nembutalized rats.

Further information on the specificity of these two compounds is provided by our experiments in which hydrocortisone was used as a blocking agent. According to Porter and Jones (5) hydrocortisone prevents ACTH discharge without interfering with hypophysial functions. Our results are in agreement with the earlier findings of Porter and Rumsfield (16) that only Pitressin is able to induce an ACTH release under these conditions. Porter and Jones (5) were the first to demonstrate that blood obtained from the hypophysial portal vessels caused an ACTH discharge from the pituitary gland in rats treated with hydrocortisone. Therefore the lipide extract obviously does not contain an ACTH-releasing factor which appears in the portal circulation.

Moreover, of both substances tested only Pitressin was still active in rats bearing hypothalamic lesions which were effective in abolishing the hypophysial stress response. A similar effect has been observed by McCann and Brobeck (24) with huge doses of Pitressin in rats bearing extensive lesions in the anterior hypothalamus. Roberts (25) reported that the lipide extract was highly active in rats bearing lesions in the posterior hypothalamus.

From these experiments one may conclude that Pitressin contains the neurotransmitter.

Another argument in favor of the nonspecificity of the lipide extract was provided by assaying this substance on surviving hypophysial tissue, which kindly was carried out for us by Dr. Saffran. The extract appeared to exert a slight but not statistically significant effect.

Further evidence for this assumption follows from the experiments of Saffran *et al.* (9) who demonstrated corticotrophin releasing activity of a Pitressin contaminant in surviving hypophysial tissue. Guillemin *et al.* (26) obtained a purified fraction from hypothalamic extracts according to the Kamm procedure for vasopressin which proved to be effective in stimulating the release of ACTH from hypophysial tissue *in vitro*.

McCann and Brobeck (24) demonstrated that compensatory adrenal hypertrophy did not occur in rats bearing lesions in the anterior hypothalamus. Therefore we decided to use the absence of the compensatory adrenal hypertrophy as a measure for the effectiveness of the lesions in abolishing the hypothalamic control of pituitary function. Moreover, in this way the possibility remained to measure at the same time the effect of several substances on the ascorbic acid content of the left adrenal gland.

The mean weight of the left adrenal gland (Table 1) indicates that the adrenals in the lesioned rats had hypertrophied already two days after the lesion had been placed. The same had been observed by McCann (29). The right adrenal weight determined one week after unilateral adrenalectomy, indicates that this hypertrophy has disappeared and that no compensatory hypertrophy has occurred. In the control group the right adrenal weight shows the presence of a significant compensatory hypertrophy.

It must be reminded, however, that in rats the left adrenal gland is distinctly heavier than the right one. This means that the differences in left and right adrenal weight are not quite comparable. In reality, the percentage decrease of the right adrenal gland in the lesioned rats is smaller, while in the control group the increase in weight is higher.

The absence of compensatory adrenal hypertrophy in the lesioned rats shows that a lesion destroying the entire median eminence, but leaving the hypophysial stalk intact, completely abolishes the stress response. The ACTH-releasing activity of Pitressin in these rats indicates that hypophysial functions are not impaired.

Morphine administered to nembutalized rats completely prevented the activity of both the lipide extract and of 0.2 U. Pitressin. The latter is in agreement with the findings of Briggs and Munson (27) using 0.07 U. vasopressin, and of Ohler and Sevy (28) who administered 0.1 U. vasopressin. However, our results do not fit in with the recent data offered by McCann (29), who stated that 0.25 U. and higher doses of Pitressin could not be antagonized by morphine. This contradiction may be best explained by assuming that the morphine blockade was less complete in McCann's experiments, witness the following facts: 1. the time interval between the intraperitoneal injection of morphine and the administration of Pitressin was only 10 minutes; 2. the hypophysis still responded to epinephrine.

The influence of morphine on the activity of Pitressin is not yet clear. In our opinion morphine might interfere with hypophysial functions by a depression of cellular metabolism.

It is still remarkable that a lipide extract from bovine posterior hypothalamus is able to release ACTH from the pituitary gland in normal and nembutalized rats. In a previous paper (30) it was demonstrated that this effect is not inhibited by dihydroergotamine, atropine, hexamethonium or neo-antergan.

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COMPARISON OF RENAL AND HEPATIC EFFECTS OF FASTING, CORTISONE ADMINISTRATION AND GLUCOSE INFUSION IN NORMAL AND ADRENALECTOMIZED RATS[1]

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ABSTRACT

The authors have measured glycogen deposition and changes in the activity of the enzyme glucose-6-phosphatase in rat liver and kidneys in several metabolic states. The administration of cortisone did not result in the accumulation of kidney glycogen, in sharp contrast to the marked and well known resulting accumulation of liver glycogen. Hyperglycemia and glucosuria, however, whether due to alloxan diabetes or to glucose infusions in the presence of a functionally intact pancreas, produced a small but significant increase in kidney glycogen. Glucose-6-phosphatase activity was found to be increased in similar fashion in both liver and kidneys of intact animals in metabolic states associated with accelerated endogenous glucose production, such as in fasting or in the diabetic state. In adrenalectomized animals this response to fasting no longer occurred. Substitution therapy with cortisone restored the normal glucose-6-phosphatase response to fasting in both organs. In discussing these results the authors present some evidence in support of the view that the enzyme glucose-6-phosphatase is not involved in the tubular reabsorption of glucose.

THE most important function of the kidney with regard to carbohydrate metabolism in the intact organism is that of preserving filtered glucose by tubular reabsorption, although the mechanism of this reabsorption is as yet unknown. In addition, it has been repeatedly suggested that the kidney may also contribute to the homeostasis of blood glucose by exerting some gluconeogenetic activity. Thus, Reinecke (1) observed that in hepatectomized animals the development of hypoglycemia was more precipitous when the kidneys were also removed, suggesting the formation and release of glucose by this organ. Goda (2) obtained direct evidence for

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the ability of renal tissue to synthesize and release glucose by demonstrating that kidney slices incubated *in vitro* with fructose rapidly converted this substrate to glucose, indeed as rapidly as did liver slices. Furthermore, similarities in the hormonal regulation of gluconeogenesis in liver and kidney have been described (3, 4, 5). Willhelmi and subsequently Teng have observed that kidney slices from adrenalectomized rats formed less glucose when incubated with alanine or pyruvate than did kidney slices from intact rats. Teng also reported increased glucose formation by kidney slices from diabetic rats incubated with pyruvate.

Liver and kidney have two more properties in common with respect to carbohydrate metabolism. In both tissues glucose-6-phosphatase activity, which is presumably necessary for the dephosphorylation of glucose-6-phosphate to glucose, is present in considerable quantity (6). Also, both tissues are capable of storing glycogen. Renal glucose-6-phosphatase activity is mainly limited to the proximal tubular cells (7), where glucose reabsorption also takes place (8); whereas glycogen accumulates principally in more distal tubular cells (9). In the present study the effects of fasting and of cortisone administration on glycogen deposition and on glucose-6-phosphatase activity have been compared in both organs.

MATERIAL AND METHODS

Animals and Experimental Groups: Male albino rats of the Wistar strain, raised in our own colony, weighing between 250 and 300 gm. were used. They were fed Purina laboratory chow ad libitum unless otherwise noted. Bilateral adrenalectomy was performed under ether anesthesia and, when experiments were carried out in adrenalectomized animals, they were performed 4 to 6 days after operation. Adrenalectomized animals were offered one per cent NaCl in place of drinking water. Diabetes was produced by the rapid intravenous injection of alloxan (40 mg. per kilogram). Animals with random blood glucose levels greater than 300 mg.% two weeks or more after the injection of alloxan were considered diabetic.

When constant infusions of glucose were to be administered operative procedures were performed under ether anesthesia without rigid aseptic technique. Polyethylene tubing (P.E. 60) was inserted into the vena cava below the entrance of the renal vein and the tubing was secured with a tie to the back muscles. The tubing was then brought through the right abdominal wall and again tied to the fascia before exteriorization in the scapular region. The animals were maintained in restraining cages (10), had access to food, and were infused with 0.6% NaCl without added heparin, at the rate of 0.1 ml. per hour for 48 hours prior to the beginning of glucose infusions. A Bird constant infusion pump was used for these experiments.

Biochemical Determinations: Animals were sacrificed by stunning and exsanguination, and the tissues to be analyzed were rapidly removed. Samples for glycogen analysis were weighed on a Roller Smith torsion balance and placed in hot 30% KOH. Glycogen was determined by the method of Good, Kramer and Somogyi (11) employing the Nelson modification (12) of the Somogyi method (13) for the measurement of glucose. Blood and urine glucose determinations were carried out according to the method of Froesch and Renold (14). Glucose-6-phosphatase activity was assayed in freshly prepared tissue homogenates by measuring the rate of release of inorganic phosphate when tissue homogenates were incubated with glucose-6-phosphate in citrate buffer at pH 6.8, as

described by Ashmore (15). Phosphorus was determined by the method of Fiske and Subbarow (16).

RESULTS

Figure 1 illustrates the deposition of glycogen by liver and kidney of adrenalectomized rats following repeated subsutaneous administration of cortisone. Liver glycogen in the untreated fasted adrenalectomized rats was 0.180 mg. per gm. of wet tissue and increased to 5.63 mg. per gm. as early as 4 hours after the injection of 20 mg. of cortisone. Hepatic glycogen

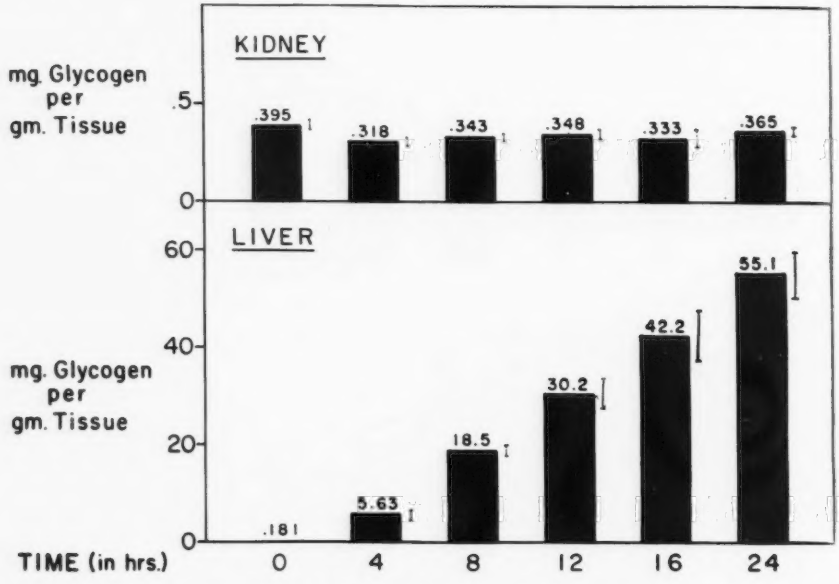

FIG. 1. Glycogen deposition in liver and kidney of adrenalectomized rats following subcutaneous administration of cortisone (20 mg. initially, then 10 mg. q 4 hours×2, and 5 mg. q 4 hours×2).

content then steadily increased in the animals sacrificed 8, 12, 16, 20 and 24 hours after the first injection of cortisone, reaching a maximal value of 55 mg. per gm. at the end of 24 hours. In contrast, no glycogen accumulated in the kidney of these same animals following the administration of cortisone. Renal glycogen varied irregularly between 0.318 and 0.395 mg. per gm. of wet tissue and there was no tendency for it to increase as a result of cortisone administration.

The activity of the enzyme glucose-6-phosphatase in liver and kidney of fed, fasted and diabetic rats is shown in Figure 2. It is apparent that liver glucose-6-phosphatase activity was increased from 5.8 to 8.6 mg. per gm. by fasting and was 10.8 mg. per gm. of tissue in the diabetic animals. Similarly, kidney glucose-6-phosphatase activity increased from 4.9 mg. per

gm. in the fed animals to 7.6 in the fasted and 10.8 mg. per gm. in the diabetic rats. All of these values are expressed in terms of mg. of inorganic phosphorus released from glucose-6-phosphate at pH 6.8.

The response of glucose-6-phosphatase activity to fasting was further investigated in adrenalectomized animals with and without cortisone administration, in doses ranging from 2.5 to 25 mg. daily. In the adrenalectomized rats, fasting did not result in increased glucose-6-phosphatase activity in either liver or kidney (Table 1). The slightly higher figure of 6.5 mg. per gm. obtained in the liver of the fasted adrenalectomized animals, when

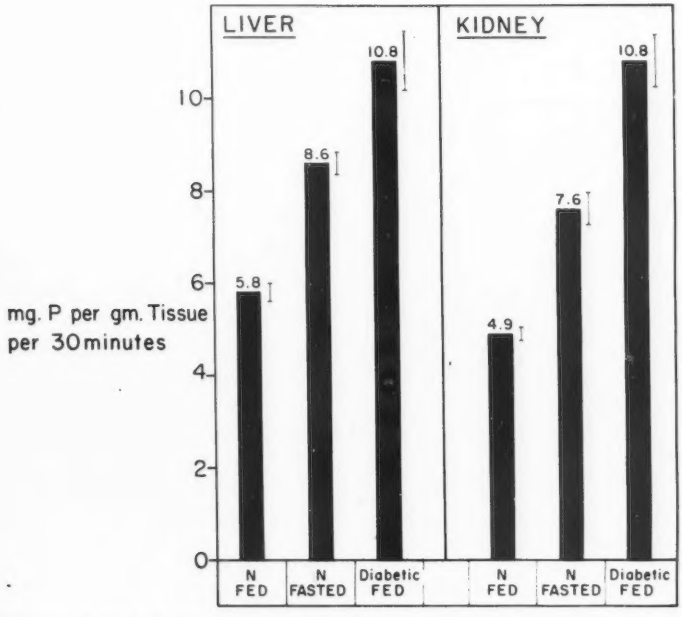

FIG. 2. Glucose-6-phosphatase activity in liver and kidney of fasted and diabetic rats. (Activity expressed as in Table 1)

compared with that of 5.7 mg. per gm. in the fed adrenalectomized rats, does not exceed that which may be attributed to the decreased total liver mass which results from fasting (15). Cortisone replacement therapy restored the normal response to fasting. Thus renal glucose-6-phosphatase activity increased to 7.3 and 8.8 mg. per gm. of tissue after a 24 hour fast in conjunction with 24 and 48 hours of cortisone administration. Hepatic glucose-6-phosphatase activity showed the normal response to fasting only in those adrenalectomized animals which had been treated with cortisone for 2 consecutive days. Cortisone administration alone, without fasting, did not significantly alter hepatic or renal glucose-6-phosphatase activity of adrenalectomized rats, with the dosages employed in this study.

TABLE 1. LIVER AND KIDNEY GLUCOSE-6-PHOSPHATASE ACTIVITY

(Glucose-6-phosphatase activity was measured in whole tissue homogenates at pH 6.8 at 30° C. The values are expressed as mg P released per gram wet tissue per 30 minutes.)

Experimental groups	Liver G-6-phosphatase			Kidney G-6-phosphatase		
	No. Obs.	mgP	±SE	No. Obs.	mgP	±SE
Normal, Fed	6	5.8	0.20	8	4.9	0.13
Normal, Fasted	13	8.6	.28	4	7.6	.35
Adx, Fed	9	5.7	.28	4	5.9	.10
Adx, Fasted	10	6.7	.27	8	5.2	.27
Adx, Fasted+24 hr. E*	10	6.5	.54	10	7.3	.39
Adx, Fasted+48 hr. E*	7	8.2	.32	7	8.8	.66
Diabetic Fed	11	10.8	.62	5	10.8	.58

* Cortisone by intramuscular injection.

A group of six intact rats was infused at a constant rate with between 10 and 22 grams of glucose for 24 and, in one case, 12 hours. The results of these experiments are summarized in Table 2, and are compared with two animals infused with 0.6% NaCl solution. In one animal the infusion of 10 gm. of glucose over 24 hours produced no definite hyperglycemia, and only 70 mg. glucose was excreted in the urine. Another animal received 12 gms. of glucose over 24 hours, exhibited blood glucose values between 190 and 230 mg.% and excreted 1.08 gm. of glucose in the urine. The remaining four animals were grossly hyperglycemic, with blood glucose values ranging from 250 to 860 mg.% and glucosuria varying between 3.10 and 8.40 gm. At sacrifice, hepatic glycogen ranged as high as 137 mg. per gm. of wet tissue. Renal glycogen varied between 0.80 and 1.47 mg. per gm. of tissue, values approximately double the normal ones. The hepatic glucose-6-

TABLE 2. EFFECT OF CONTINUOUS GLUCOSE INFUSION ON GLYCOGEN DEPOSITION AND GLUCOSE-6-PHOSPHATASE ACTIVITY IN LIVER AND KIDNEY

(Activity expressed as in Table 1)

Rat number	1	2	3	4	5	6	7	8
Total Glucose Infused (in grams)	0	0	11	10	12	18	18	22
Duration of Glucose Infusion (Hours)	—	—	12	24	24	24	24	24
Total Glucose Excreted (in grams)	0	0	3.1	0.07	1.08	4.74	5.41	8.41
Blood Glucose at Sacrifice (mg.%)	80	87	366	120	232	420	515	860
Kidney Glycogen (mg./gm)	0.49	0.47	0.90	1.03	1.47	1.05	0.96	0.80
Liver Glycogen (mg./gm.)	22	14.5	96.2	—	106	137	117	132
G-6-Phosphatase Activity								
Per Gram Liver (mg. P/gm./30′)	8.3	7.0	5.1	4.1	3.0	3.7	4.8	4.2
Per Total Liver (mg. P/30′)	72	62	80	53	43	61	69	67
Per Gram Kidney (mg. P/gm./30′ um)	6.4	7.5	6.5	6.6	5.4	6.7	5.1	5.7

phosphatase activity of the glucose-infused animals was significantly lower (4.1 mg. inorganic phosphorus released per gm. of wet liver), than that of either normal fed animals (Table 1) or that of the two animals infused with saline alone. The apparent elevation in glucose-6-phosphatase in the two restrained and infused animals not receiving glucose may be due to partial fasting under restraining conditions. Total liver glucose-6-phosphatase activity appeared to be slightly lowered only in the two animals receiving 10 and 12 gm. of glucose per day over a 24 hour period. Kidney glucose-6-phosphatase activity did not appear to be altered by the hyperglycemia and glucosuria resulting from glucose infusions.

DISCUSSION

It has been suggested by Ashmore (17) and Weber (18) that the activity of the hepatic enzyme glucose-6-phosphatase is increased in states associated with active hepatic gluconeogenesis. Thus, glucose-6-phosphatase activity is increased in diabetes mellitus and in steriod-induced diabetes and, to a lesser degree, in fasting. It would appear from the studies reported here that changes in renal glucose-6-phosphatase activity induced by fasting and by the diabetic state closely parallel those observed in the liver. Furthermore, in both organs, the increase of glucose-6-phosphatase activity due to fasting was abolished by adrenalectomy, and was restored by therapy with cortisone. Since the magnitude of the rise in the activity of this enzyme under fasting conditions was not influenced by the dose of cortisone administration, it seems reasonable to assume that cortisone played a "permissive" (19) role in this response to fasting.

The accumulation of glycogen in the liver of the fasted adrenalectomized rat after glucocorticoid administration is well known and has even been used for bioassay procedures (20). It is evident from the results reported in Figure 1 that glycogen deposition in the kidney is not subject to the same hormonal regulation. Increased renal glycogen levels were measured only in diabetic animals and in the animals infused with glucose. The accumulation of glycogen in the tubules of diabetic animals has been attributed either to lack of insulin or to prolonged glucosuria (21). The results shown in Table 2 would tend to favor the second hypothesis since increased levels of glycogen were found in the kidneys of rats made hyperglycemic and glucosuric by the infusion of glucose, despite a presumably normal response of the insulin mechanism.

It is of interest, to note that the activity of glucose-6-phosphatase in the kidney is increased in hyperglycemic-glucosuric diabetic rats and normal in rats in which hyperglycemia and glucosuria was produced by the infusion of glucose. These two states differ in that gluconeogenesis is accelerated in diabetic animals whereas it most likely proceeds at a slow rate in normal animals upon which hyperglycemia has been forced by means of a

glucose infusion. Again, changes in the activity of glucose-6-phosphatase seem to correlate with the rate at which its substrate, glucose-6-phosphate, is presented for dephosphorylation. At this point a further question arises. If the formation of glucose-6-phosphate were involved in the process of glucose reabsorption by the renal tubular cells, a large glucose load to the tubules should be expected to lead to a rise in glucose-6-phosphatase activity. The demonstrated lack of such a response suggests that the enzyme glucose-6-phosphatase may not be involved in the tubular reabsorption of glucose. Evidence against the phosphorylation theory of glucose reabsorption has also been gathered by Mudge and Taggart (22), Cori (23) and Dratz and Handler (24) and a different mechanism has been proposed by Keston (25).

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ADRENAL CORTICAL REGULATION OF HEPATIC GLUCOSE METABOLISM[1]

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ABSTRACT

In order to further study the role of adrenal cortical steroids on the regulation of hepatic glucose metabolism, liver slices from normal, alloxan-diabetic, adrenalectomized, adrenalectomized diabetic and hydrocortisone treated rats were incubated with differentially labeled C^{14} glucose and fructose. From such studies the rate of glucose phosphorylation and the metabolism of glucose via the phosphogluconate oxidation pathway have been calculated. Our results would indicate that the adrenal cortical steroids effect neither of these functions. The only effects observed were those of increased gluconeogenesis and decreased lipogenesis.

THE role of adrenal cortical hormones in the regulation of hepatic carbohydrate metabolism has been widely studied from the standpoint of gluconeogenesis and glycogen deposition. Many of the alterations in hepatic carbohydrate metabolism observed in diabetes are returned toward normal following adrenalectomy (1). Specifically these include a reduction in gluconeogenesis and in glucose-6-phosphatase activity and an increase in lipogenesis (2, 3). However, relatively little is known of the influence of glucocorticoids on glucose phosphorylation and pathways of glucose-6-phosphate metabolism. We have previously compared in liver slices from normal and alloxan-diabetic rats the relative rates of glucose-6-phosphate metabolism via the alternate routes available in heaptic tissue (4). In this work these studies have been extended to include the effects of glucocorticoids on pathways of glucose metabolism.

METHODS

Animals.—Male albino rats of the Wistar strain, weighing between 250 and 350 gm. were used. All animals were fed *ad libitum* on Purine laboratory chow. Alloxan diabetes was produced by intravenous injection of alloxan monohydrate (40 mg. per kilo) as previously described. Only animals which had blood sugar greater than 300 mg.% and in which at least 15 days had elapsed after alloxan injection were used. Bilateral adrenalectomy was performed under ether anesthesia and animals were used 4 to 6 days after operation. Adrenalectomized rats were maintained on 1% NaCl *ad libitum*. Rats receiv-

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ing 17 hydroxycorticosterone (hydrocortisone) were injected (subcutaneously 5 mg. per 12 hours) over a period of 5 days.

Medium and substrates.—Animals were killed by a blow on the head, the liver rapidly removed, chilled and sliced with a Stadie-Riggs slicer. Tissues slices were weighed on a Roller-Smith torsion balance and placed in oxygenated buffer. Hastings high potassium medium was used for all incubations. Glucose-1-C^{14}, glucose-6-C^{14}, glucose-U-C^{14}, fructose-U-C^{14} and pyruvate-2-C^{14} were employed as labeled substrates. When glucose and fructose were used simultaneously, their concentrations were each 15 Mmoles per liter, only one of the two substrates being labeled with C^{14} in any one flask (5). Pyruvate was incubated alone at a concentration of 40 Mmoles per liter.

Analyses.—The liver slices were analyzed for glycogen (6) and the media for glucose (7), fructose (8), pyruvate (9), and CO_2 (10). Glucose and fructose were isolated as their phenylosazone and assayed for C^{14}. CO_2 was collected as $BaCO_3$, for plating and counting. Pyruvate was precipitated as the dinitrophenyl hydrazone for C^{14} assay. These procedures have been described previously.

Calculations.—The following derived data have been obtained from experimental measurements by the methods of calculation previously described (4): (1) glucose to glycogen, (2) glucose oxidized to CO_2, (3) glucose phosphorylation, (4) glycogen formation from fructose, (5) glucose formation from fructose, (6) fructose oxidized to CO_2, (7) glucose metabolism via the phosphogluconate oxidation pathway, (8) glucose phosphorylated to glucose-6-phosphate then directly hydrolyzed to glucose and inorganic phosphate. All values have been expressed in micromoles per gm. of wet liver per 90 minutes.

RESULTS

In order to assess the role of glucocorticoids on hepatic glucose metabolism, liver slices from normal, alloxan-diabetic adrenalectomized, adrenalectomized diabetic and rats treated for 5 days with excessive quantities of hydrocortisone have been compared (Table 1.) Both glucose and fructose have been used as substrates since such a combination allows one to calculate the amount of glucose phosphorylated. The results obtained with the various animal preparations have been compared with the normal and alloxan-diabetic values which are summarized in Table 1.

Adrenalectomized animals.—Values for glucose and fructose metabolism by liver slices from adrenalectomized rats compare roughly to those obtained with normal animals (Table 1). There is no evidence of any defect in the ability of hepatic tissue of adrenalectomized animals to oxidize glucose or store it as glycogen. Fructose is utilized at a normal rate and carbon from this labeled substrate is incorporated into glucose, glycogen and CO_2 at a normal rate.

Adrenalectomized diabetic.—The incorporation fo C^{14} from labeled glucose into glycogen by slices from adrenalectomized diabetic animals is slightly greater than that found in alloxan-diabetic animals (Table 1), but is still $\frac{1}{3}$ that of normal. It is difficult to say whether the rate of oxidation of carbon-1 of glucose has been altered since this appears to occur to about the same extent in normal and diabetic slices. The decreased incorporation of

TABLE 1. METABOLISM OF GLUCOSE (15 MMOLES PER LITER) AND FRUCTOSE (15 MMOLES PER LITER) BY RAT LIVER SLICES
(All values expressed as micromoles of glucose or fructose per gm. wet liver per 90 min.)

		Glucose				Fructose				
	No. obs.	Label	Net change	To glycogen	To CO_2	Label	Uptake	To glucose	To glycogen	To CO_2
Normal Rats										
Mean	(6)	1-C^{14}	58.0	7.5	0.88	U-C^{14}	67.0	27.0	9.0	5.3
±S.E.			10.1	1.04	0.65		3.7	2.1	1.3	0.36
Mean	(4)	6-C^{14}	63.0	6.1	0.31		68.0	29.0	10.0	5.4
±S.E.			4.3	1.5	0.06		7.0	—	2.2	0.4
Alloxan Diabetic Rats										
Mean	(6)	1-C^{14}	94.0	0.21	0.58		86.0	41.0	1.5	5.6
±S.E.			7.5	0.03	0.05		1.9	3.9	0.42	0.51
	(3)	6-C^{14}	103	0.32	0.31		84	42	2.0	5.5
Adrenalectomized Rats										
Mean	(5)	1-C^{14}	46	7.4	1.7		71	37	16	9.0
±S.E.			5.0	2.3	.2		3.1	6.5	3.7	.7
	(2)	6-C^{14}	56	3.7	.6		74	40	14	8.6
Adrenalectomized Diabetic Rats										
Mean	(5)	1-C^{14}	65	1.5	1.3		64	47	9.7	7.3
±S.E.			14	.48	.01		6.0	9.3	1.8	.77
	(2)	6-C^{14}	45	1.4	0.3		54	36	7.0	6.0
Hydrocortisone Treated Rats										
Mean	(6)	U-C^{14}	91	2.1	0.98		68	37	2.1	6.7
±S.E.			6.0	.04	—		10	—	.04	—
Mean	(6)	1-C^{14}	91	1.9	.96		58	26	2.3	6.1
±S.E.			3.5	.14	.02		.37	6.5	.19	.34
Mean	(6)	6-C^{14}	92	2.4	.60		58	26	2.3	6.1
±S.E.			4.2	.10	.12		.37	6.5	.19	.34

carbon from fructose into glycogen observed in the diabetic (reduced from 9 to 1.5 μM/gm.) was not observed in the adrenalectomized diabetic.

Hydrocortisone treated.—In liver slices from rats treated with hydrocortisone over a period of 5 days, glucose production by the isolated tissue is increased 60% over normal and is similar in degree to that observed in diabetic animals. Glycogen synthesis from both glucose and fructose is markedly reduced in liver slices from these animals. Such a decrease may be due in part to an increase in glucose-6-phosphatase activity. Although net glucose production has been increased, the conversion of fructose to glucose does not appear to have been accelerated.

Glucose phosphorylation.—From the data presented in Table 1, it is possible to calculate maximum and minimum rate sof glucose phosphorylation. Such values calculated for the various animal preparations are summarized in Table 2. A significant reduction in glucose phosphorylation occurs only in the diabetic and diabetic adrenalectomized rats.

TABLE 2. GLUCOSE PHOSPHORYLATION BY LIVER SLICES
(All values expressed as μM/gm./90 min.)

	No. obs.	Max.	Min.	Mean
Normal	6	55	29	42
Diabetic	6	13	6.3	9.6
Adrenalectomized	5	33	25	29
Adrenalectomized diabetic	5	9.9	8.7	9.3
Hydrocortisone injected	6	48	23	36

TABLE 3. METABOLISM OF PYRUVATE-2-C^{14} (40 MMOLES PER LITER) IN RAT LIVER SLICES
(All values expressed as micromoles of Pyruvate per gm. wet liver per 90 min.)

Net glucose	Pyruvate to		
	Glucose	Glycogen	Fatty acids
	Normal Rats		
58	9	17	41
82	23	12	46
81	16	9	50
Mean 74	16	13	46
	Alloxan Diabetic Rats		
94	52	4.0	1
131	54	1.6	4
82	40	2.4	4
Mean 102	48	2.4	3
	Adrenalectomized Diabetic Rats		
62	14	9	15
67	12	15	51
62	29	14	16
62	25	11	29
67	31	21	30
Mean 64	22	14	24

Pyruvate metabolism.—Although the metabolism of pyruvate by liver slices from normal, diabetic, and adrenalectomized diabetic rats has previously been compared, these data have been briefly summarized (Table 3.). Incorporation of C^{14} from pyruvate into glucose is increased 3 fold in the diabetic and is returned to normal in the adrenalectomized diabetic.

DISCUSSION

Glucose Utilization.—Previous studies by Renold *et al.* (11) have demonstrated that glucose utilization by liver slices from adrenalectomized rats was at a normal rate and that slices from adrenalectomized diabetic rats used glucose at a rate similar to those from diabetic animals. It was also demonstrated that liver slices from rats pre-treated for several days with large doses of cortisone used glucose at a normal rate (12). Since hepatic cells are able to hydrolyze glucose-6-phosphate and liberate free glucose, the glucose uptake by the slice may be influenced by both rate of phosphorylation of glucose and rate of dephosphorylation. Adrenal cortical steroids have been shown to increase the activity of hepatic glucose-6-phosphatase; this might be presumed to increase the rate of glucose-6-phosphate hydrolysis which in turn would reduce the glucose uptake. For this reason glucose phosphorylation was studied. The results summarized in Table 2 indicate that adrenal cortical hormones do not alter glucose phosphorylation by hepatic tissue. In contrast, glucose utilization by isolated rat diaphragm muscle may be subject to some adrenal cortical regulation (13, 14, 15) although administration of large doses of cortisone to intact rats does not impair glucose utilization (16).

Glucose oxidation.—Since the liver is one of the several tissues that contain an active hexose-phosphate oxidation pathway (17) it is of interest to examine the extent of adrenal regulation of hexose phosphate metabolism via this route. The maximum quantity of C-1-labeled glucose that can be metabolized via this pathway can not exceed the μM of $C^{14}O_2$ occuring during incubation (10). A minimum value can be calculated from the difference in oxidation of carbon-1 and carbon-6 of glucose (10). Such calculations (Table 4) reveal that under the conditions studied a maximum of 1 to 2 μM of glucose are metabolized by the direct oxidative route. The contribution of this pathway to glucose-6-phosphate metabolism appears rather small and is not markedly changed by glucocorticoids.

Glycogen synthesis.—The *in vitro* synthesis of glycogen from glucose, fructose and pyruvate appears to be reduced only in the livers from alloxan-diabetic and hydrocortisone treated animals. Utilization of both fructose and pyruvate is essentially the same in all preparations studied and reduced glucose phosphorylation was not observed in hydrocortisone treated rats. It therefore appears that the reduced glycogen synthesis observed could not be the result of an alteration in substrate utilization. However, glucose-6-phosphatase activity is increased in livers of diabetic and hydrocortisone treated animals and is normal in the adrenalectomized and adrenalectomized-diabetic animals (2). This suggests that the reduced glycogen synthesis observed may be due to an increase in the activity of this enzyme.

Glucose formation.—An increase in net glucose production and pyruvate to glucose (Table 3) observed in liver slices from alloxan-diabetic rats is returned to normal following adrenalectomy of the diabetic (2). An increase in glucose formation by liver slices from adrenalectomized diabetic animals may be observed by the administration of hydrocortisone to such animals several hours prior to sacrifice. Welt *et al.* (16) have calculated hepatic glucose production in intact normal, diabetic and cortisone treated rats. Their results indicate a twofold increase in glucose production in the diabetic and a sevenfold increase in the cortisone treated animals. Similar results have been obtained with labeled pyruvate in this study. A threefold increase in pyruvate to glucose was observed in the diabetic.

Role of insulin.—Although we have been concerned mainly with the function of the adrenal cortical steroids in the regulation of hepatic carbo-

TABLE 4. GLUCOSE METABOLIZED VIA THE PHOSPHOGLUCONATE OXIDATION PATHWAY
(All values expressed as μM glucose/gm. liver/90 min.)

	Max.	Min.
Normal	0.9	0.6
Diabetic	0.6	0.3
Adrenalectomized	1.7	1.1
Adrenalectomized diabetic	1.3	1.0
Hydrocortisone treated	1.0	0.4

hydrate metabolism, it is apparent that these studies are also pertinent to the role of insulin in regulation of liver metabolism. For example, the decreased rate of glucose phosphorylation observed only in diabetic and adrenalectomized diabetic rats suggests that insulin is the hormone responsible for the regulation of this function. The regulation of hepatic glucose production appears to be primarily the function of the adrenal cortical steroids with insulin acting in opposition to the steroid effect. It is also apparent that both insulin and the adrenal steroids influence lipogenesis, but in opposite directions. Whether or not the insulin effects on gluconeogenesis and lipogenesis are primary insulin effects, or are secondary to the action of insulin on glucose phosphorylation is not clear.

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INHIBITION OF GLUCAGON AFTER *IN VITRO* MIXTURE WITH LITHOSPERM

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ABSTRACT

The hyperglycemic effect of glucagon in the fowl was inhibited after its mixture *in vitro* with cold-water extracts of the plant *Lithospermum ruderale*. Spray-dried powders prepared from the cold-water extracts also inhibited glucagon activity after *in vitro* mixture with the hormone. A preliminary fractionation of the spray-dried powders by paper-column chromatography yielded several fractions, some of which were active. The active fractions were eluted by water from the column. The *in vitro* inhibition of glucagon appears to parallel the "inactivation" of pregnant mare serum and other protein hormones which follow *in vitro* mixture with lithosperm.

Very high dosages of the spray-dried lithosperm powder given either subcutaneously or intravenously did not produce hypoglycemia in pullets, cockerels, or laying hens. It is concluded that *in vitro* inactivation of glucagon occurred in the experiments reported.

KEMPER, Loeser, Opitz, and Schwartz (1) have recently reported that the administration of extracts of *Lithospermum officinale* lowered blood-sugar level 45% to 55% in 3 out of 5 rabbits tested. This suggests that extracts of *Lithospermum* may affect carbohydrate metabolism and adds another item to the list of physiological mechanisms reported to be influenced by extracts of this plant. Accordingly, some of our observations relative to the inhibition of glucagon activity by extracts of *Lithospermum ruderale* may contribute to a better understanding of this latest phenomenon.

The *in vitro* assay procedure for lithosperm[3] activity developed by Noble and his co-workers (2, 3, 4) has been used for screening numerous chemical fractions. Since *in vitro* mixtures have been reported by Noble to result in an inactivation of pregnant mare serum, interstitial cell-stimulating hormone, luteotrophic hormone, and thyrotrophin, we wished to determine if our lithosperm extracts affected these and other protein hormones. Atten-

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[3] The term lithosperm is used as a convenient generic designation for the hormone inhibiting principle or principles present in any members of the genus *Lithospermum* without committment as to their chemical identity.

tion was first directed to insulin because its molecular structure and amino-acid composition are known. It was hoped that if lithosperm inactivated this hormone it might be possible to determine the exact nature of the inhibitory process. The interesting results of our first lithosperm-insulin mixtures and the availability of both amorphous and crystalline glucagon[4] subsequently led us to extend our investigations to the latter hormone.

MATERIALS AND METHODS

Single-comb White Leghorn cockerels and pullets from 40 to 90 days-of-age were used as test animals in all but one experiment. The procedures followed were those outlined by Beekman (5). The birds were fasted for twelve hours, weighed, and than allowed to feed for four hours before administration of test solutions. The amounts of insulin and glucagon injected were on the basis of kilograms of body weight of the fasted animals. The lithosperm extract in the preliminary insulin experiments was mixed with the hormone and incubated at 37° C for two hours. Incubation was omitted in the glucagon experiments because it was discovered that a change in hormone activity would occur without incubation after mixture for only one hour. The volume of the lithosperm-hormone mixtures was 1.0 ml. The lithosperm preparations were approximately pH 7.0, therefore, it was necessary to adjust the lithosperm-glucagon mixtures with glycine buffer to a pH of 9.5 or 10.0.

Since the amount of insulin-lithosperm or glucagon-lithosperm mixture received by the birds was adjusted for body weight, the quantity injected intramuscularly usually was only 0.15 ml. Blood samples were taken for glucose determinations both before injection and, except in the insulin experiment, one hour after the administration of the solutions. The blood-glucose determinations were made according to the Folin and Malmros micro-method, Horvath and Knehr (6).

Dried whole overground parts of *Lithospermum ruderale* secured from commercial suppliers were used as the starting material. The dried plant was finely ground in a Wiley laboratory grinder and was usually powdered still finer in a hammer-mill. Extracts were made at room temperature with 10 parts of deoxygenated water per part of plant. Several successive extractions generally were made and combined for further processing. The best material was obtained if the first washings were not combined with the bulk of material to be worked up. Filtration of the mass was very difficult and was most successfully achieved in thin layers, with the aid of pressure and with filter aids such as Supercel. The clarified solutions were evaporated as rapidly and gently as possible under reduced pressure. A combined procedure was advantageous, involving a partial concentration in an efficient flash-evaporator followed by a complete drying to powder in a spray-drying apparatus. As an alternative, a freeze-drying technique may be used instead of the spray-drying to remove water. The water-soluble constituents of the powder prepared as described contain some organic and inorganic salts and probably also saccharides (they ferment rapidly and support the growth of molds).

Several methods have been employed for the fractionation of extract powders, but only one preliminary experiment on fractionation is included in this report. An early effort was made to separate the constituents by means of chromatography on columns packed with cellulose powder. The chromatography of 10 grams of an active lithosperm powder was conducted on a column four inches in diameter containing one kilogram of Whatman Standard Grade of cellulose powder. The extract was mixed with a small amount of cellulose powder and carefully placed on the top of the packed column after

[4] The glucagon used in this study was supplied through the courtesy of Eli Lilly and Co., Indianapolis, Indiana.

which the initial eluting solvent, 3:1 acetone-water was added. The solvent was gradually changed to pure water by the slow addition of water. The fractions used for the data in Table 5 were prepared in this manner.

RESULTS AND DISCUSSION

It may seem unusual to employ the fowl instead of a mammal as a test animal for these studies, especially since the cat is excellent for glucagon assay. Beekman (7, and unpublished data) has shown that blood-sugar of the fowl will be elevated 45% by as little as 0.1 μgm. of crystalline glucagon. Staub and Behrens (8), on the other hand, state that the cat requires 0.2 μgm. for this increase. Furthermore, the maximum response in the fowl is not reached until at least 25.0 μgm. of glucagon is given whereas a maximum response is achieved in the cat with 1.0 μgm. to 2.0 μgm. The log of glucagon dosage against the rise in blood-sugar in the fowl is a straight line from 0.2 μgm. to 50.0 μgm. per kilogram of body weight. The

TABLE 1. EFFECT OF INSULIN AND INSULIN PLUS LITHOSPERM* ON BLOOD-SUGAR OF WHITE LEGHORN FOWL
(Percentage change 90 minutes after administration)

Insulin	Lithosperm	No. of animals	% change blood-sugar
6.7 units insulin plus 250.0 mg. eq.†		2	−21.9
6.7 units insulin plus 500.0 mg. eq.		2	−46.1
1.0 unit/kg. body weight		2	−20.7

* Incubated for 2 hours at 37° C. Each bird received 1.0 unit per kilogram of body weight thus the injection volume was about 0.15 ml. of the lithosperm-insulin mixture.
† Milligram equivalent of the original dried plant.

responses are remarkably reproducible as will be illustrated by the glucagon controls in Tables 2 and 4 of this paper. Finally, it has been reported by Vuysteke and De Duve (9) that the glucagon content of the bird pancreas is six times greater than that of the mammalian pancreas. Since we are interested in analyzing the possible effects of lithosperm on both endogenous and exogenous glucagon, the fowl is especially advantageous for our studies.

Table 1 presents the blood-sugar changes which accompanied the administration of insulin alone and following its incubation for two hours at 37° C with lithosperm. Although these data are very limited, they indicate that lithosperm did not inhibit insulin action and this result is in harmony with the observations of Noble *et al.* (2). For this reason, we did not wish to extend these experiments at this time. We were interested, however, in the observation that there appeared to be a greater decrease in blood-sugar level in those birds given the 500.0 mg. eq. lithosperm-insulin mixture than in those animals which received insulin alone. Two possible explanations for this observation were suggested: first, that there might be a trace of glucagon in the insulin preparation and that the action of this exogenous glucagon might be inhibited; or second, that the 75.0 mg. eq. of lithosperm

which was injected (0.15 ml. or 15% of the 500.0 mg. eq. in the mixture) might inhibit endogenous glucagon. Accordingly, it was decided to test directly for possible *in vitro* inhibition of glucagon and also to examine the possibility that lithosperm might produce hypoglycemia by its action *in vivo*. In the first experiments, various concentrations of a cold-water extract of dried plant were mixed with glucagon *in vitro*. These data are presented in Table 2.

The dosage of amorphous glucagon in this and all subsequent experi-

TABLE 2. DECREASED HYPERGLYCEMIC ACTION OF GLUCAGON AFTER MIXTURE *in vitro* WITH LITHOSPERM PREPARED AS A COLD WATER EXTRACT OF DRIED PLANT MATERIAL

	Glucagon	Lithosperm	No. of animals	% increase blood sugar	% decrease in activity
A*	500.0 μgm. plus	1.0 mg. eq. lithosperm‡	6	+ 54	−44.3
	500.0 μgm. plus	10.0 mg. eq. lithosperm	6	+ 38	−62.5
	500.0 μgm. plus	100.0 mg. eq. lithosperm	6	+ 13	−83.5
	75.0 μgm./kg. of body weight		6	+ 97	
B†	500.0 μgm. plus	0.5 mg. eq. lithosperm	3	+ 78	−23.6
	500.0 μgm. plus	5.0 mg. eq. lithosperm	3	+ 68	−34.4
	500.0 μgm. plus	25.0 mg. eq. lithosperm	3	+ 22	−78.4
	75.0 μgm./kg. of body weight		2	+102	

* Mixed at room temperature for 2 hours before injection
† Mixed at room temperature for 1 hour before injection.
Injection volume was about 0.15 ml. for the lithosperm-glucagon mixtures so that each bird received 75.0 μgm. of glucagon per kilogram of body weight.
‡ The dosage of lithosperm is in terms of milligrams equivalent of the original dried plant.

ments was 75.0 μgm./kg. of body weight. This amount of glucagon is sufficient to produce approximately a 100% increase in the blood-sugar level of the fowl, a fact demonstrated by the figures for the two control series in Table 2 in which the glucagon produced increases of 97% and 102% in the blood-sugar. The two groups of treatments in A and B are not exactly comparable experiments. They were performed at different times and the lithosperm-glucagon mixture was allowed to stand two hours before injection in one series and only one hour in the other. Neither mixture was incubated. Remarkably good correlation existed, however, between the amount of lithosperm used and the percentage decrease in glucagon activity which was observed. The mixture of the glucagon with as little as 0.5 mg. eq. of lithosperm had a slight but definite inhibitory effect on the hyperglycemic action of the glucagon. Except for the slight reversal in the degree of inhibition produced by the 1.0 mg. eq. and the 5.0 mg. eq. dosages, the results were remarkably constant. This reversal of effect will be commented on later. In general, inhibition was greater as the dosage of lithosperm increased. Furthermore, the 0.5 mg. eq. and the 1.0 mg. eq. of the lithosperm-glucagon mixtures were clear and almost colorless. There was no evidence of precipitation. We believe that this is significant since it has been suggested that *in vitro* mixture of lithosperm with other hor

mones might be effective as a result of precipitation of the hormone. The data in Table 2 demonstrate that glucagon action was inhibited by the cold-water extract of lithosperm after mixture at room temperature for two hours in the case of experiment A, or after mixture for only one hour at room temperature in experiment B. The results show that it is not necessary to incubate the mixtures, which avoids the possibility of heat inactivation of the hormone.

The light brown powders, prepared under the mildest conditions described earlier, keep well in cold-storage and dissolve readily in water with little or no insoluble residue. Tests for biological activity of these powders proved them to be low in toxicity and very potent in the inactivation of pregnant mare serum. Such a powder prepared for us by spray-drying by Dr. Bryce Douglas at the Smith, Kline and French Laboratories has been used as a reference substance and has retained its activity for more than a year. It was decided, therefore, to use this powder for three purposes: first, to test whether it would produce hypoglycemia per se; and second, as a starting material for the possible fractionation of lithosperm by paper-column chromatography. A few representative experiments designed to test the possible hypoglycemic effect of this lithosperm powder *in vivo* are shown in Table 3.

TABLE 3. BLOOD-SUGAR DETERMINATION ON FOWL GIVEN LITHOSPERM

No. of animals	Animal	Treatment	Blood-sugar
		Subcutaneous administration	
2	Pullets	Control	173 mg. %
2	Pullets	2.5 mg. lithosperm 2× daily—4 days	177 mg. %
2	Pullets	5.0 mg. lithosperm 2× daily—4 days	188 mg. %
2	Pullets	10.0 mg. lithosperm 2× daily—4 days	183 mg. %
3	Pullets	20.0 mg. lithosperm 2× daily—4 days	176 mg. %
4	Laying hens	Controls	160 mg. %
4	Laying hens	250.0 mg. lithosperm 2× daily—5 days	161 mg. %
		Intravenous administration	
2	Cockerels	Control	175 mg. %
2	Cockerels	20.0 mg. lithosperm	178 mg. %
2	Cockerels	20.0 mg. lithosperm	181 mg. %
2	Cockerels	20.0 mg. lithosperm subcutaneously followed 40 minutes later by 20.0 mg. lithosperm intravenously	177 mg. %

			Blood-sugar change minutes after injection						
			20	40	60	90	120	150	180
2	Cockerels	Controls	−1%	−8%	−12%	−20%	−7.5%	−8%	−5.5%
2	Cockerels	33.3 mg. LSPM/kg. intravenously	−5.5%	−4.5%	−15%	−16%	−3.5%	3%	11.5%

It will be noted that a wide range of dosages from 2.5 mg. to 250.0 mg.

were used and that the birds received amounts of lithosperm which in most instances exceeded those injected when lithosperm-glucagon combinations were given after *in vitro* mixture. For example, the birds which received the maximum amount of lithosperm in Table 2 actually were given only 15.0 mg. eq. of lithosperm since they received 0.15 ml. of the lithosperm-glucagon mixture (500.0 μgm. glucagon plus 100 mg. eq. of lithosperm). Injections were made subcutaneously and with the supposition that subcutaneous injections given twice daily might provide a rather constant level of lithosperm in the blood stream. The lithosperm was also administered intravenously in a few instances.

Pullets which received from 5.0 mg. to 40.0 mg. daily of lithosperm subcutaneously did not demonstrate any hypoglycemic response to the lithosperm. In order to test the possibility that a higher dosage of lithosperm might be necessary to produce a hypoglycemic effect, 250.0 mg. of lithosperm was injected subcutaneously twice daily into laying hens for five days. The blood-sugar level in these birds also was not altered. It is interesting, however, that egg-laying ceased in these hens after the first day of treatment and at the end of five days the ovaries and oviducts had regressed to the non-laying condition.

The route of administration was checked next. It seemed possible that lithosperm given by intravenous injection might act more rapidly and be effective. Cockerels which received lithosperm intravenously, and by both intravenous and subcutaneous injection, however, did not have their blood-sugar levels significantly changed. The possibility that the effect of lithosperm could be very short lasting and, therefore, could be missed when blood samples were taken only after 60 minutes was the next point to be checked. Cockerels were injected intravenously with lithosperm and blood samples of both control and treated birds were taken every 20 minutes for three hours. There was a slight decline in the blood-sugar of lithosperm injected cockerels during the first two hours, but the controls showed an equal or even greater drop during the same periods. It must be concluded from the data in Table 3 that under the conditions of these experiments using a spray-dried powder over a wide range of dosages that hypoglycemia is not produced in the fowl by our present lithosperm perparation.

Since it is possible that the glucagon inhibiting activity of the lithosperm might be lost by the spray-drying, attention was next directed to the study of the effect of mixing this material with glucagon *in vitro*. The data shown in Table 4 are for a series of birds which received injections of glucagon after its mixture with small amounts of spray-dried powder for one hour at room temperature. The dosages ranged from a low of 0.01 mg. to 5.00 mg. of powder but the amount of glucagon, 75.0 μgm./kg. of body weight, remained the same.

Attention should be directed to the 98.5% increase in blood-sugar in

the glucagon control series. This value is very close to the 97% and 102% figures for the controls in Table 2. The inhibition of glucagon activity by lithosperm powder is evident at all of the dosages used. More experiments, of course, should be performed before much significance can be ascribed to the results of the 0.01 mg. and the 0.10 mg. treatment levels. Additional tests also should be done to ascertain if there is a difference between the 3.00 mg. and the 4.00 mg. dosages. It is of some importance to note, however, that Graham and Noble (4) have observed that high and low dosages of some lithosperm preparations are more potent inhibitors of PMS activity than are dosages in the middle range. It will be recalled that there also was an indication of reversal in the degree of response between the 1.0 mg.

TABLE 4. DECREASED HYPERGLYCEMIC ACTION OF GLUCAGON AFTER MIXTURE *in vitro* WITH SPRAY-DRIED LITHOSPERM POWDER PREPARED FROM A COLD WATER EXTRACT

Glucagon	Lithosperm	No. of animals	% increase blood-sugar	% decrease in activity
500.0 μgm.	plus 0.01 mg. lithosperm*	10	+72.9	−25.9
500.0 μgm.	plus 0.10 mg. lithosperm	10	+77.0	−21.8
500.0 μgm.	plus 1.00 mg. lithosperm	5	+67.8	−31.2
500.0 μgm.	plus 3.00 mg. lithosperm	5	+39.0	−60.4
500.0 μgm.	plus 4.00 mg. lithosperm	5	+39.0	−60.4
500.0 μgm.	plus 5.00 mg. lithosperm	10	+12.5	−86.3
75.0 μgm./kg. of body weight		25	+98.5	

* Mixed at room temperature 1 hour before injection.
Injection volume was about 0.15 ml. for the lithosperm-glucagon mixture so that each bird received 75.0 μgm. of glucagon per kilogram of body weight.

eq. and the 5.0 mg. eq. dosages in Table 2. It may be possible that additional data will demonstrate a phenomenon here which is similar to the PMS inhibition. Regardless of the vagaries of the dosage responses, it is evident that inhibition of glucagon action does occur after its *in vitro* mixture with the spray-dried powder. The 5.00 mg. of the powder actually produced a slightly greater inhibition than did the 100.0 mg. eq. of the cold-water extract. The 3.00 mg. and the 4.00 mg. amounts compare favorably in their action with the 10.0 mg. eq. and the 25.0 mg. eq. quantities of the cold-water extract. When it is remembered that the birds injected with the 1.00 mg. lithosperm-glucagon mixture actually received only about 0.15 mg. of lithosperm, it is difficult to see how the 31.2% decrease in response could be accounted for by hypoglycemia produced *in vivo*.

The stability of the spray-dried powder and the evidence that it also would inhibit glucagon action after mixture *in vitro*, encouraged us to attempt some preliminary fractionations on a paper-column. The plant powder constituents were divided by the paper-column into a number of broad bands which showed differences in gross properties. The only bands tested in this experiment were those which we designated as one to five: with one being the 3:1 acetone-water soluble material which was the first

to pass through the column. Approximately 20 fractions were tested in all and the data presented in Table 5 were selected as representative of the results. Unfortunately, the experiments were not as critical as they might have been because of two factors: first, we were forced to use older birds (90 days-of-age) which do not respond as well as younger birds; and second, the glucagon was in solution for two weeks before use. In spite of these deviations in procedure, the results clearly demonstrate that there is a difference in the rate at which the active principle migrates through the paper-column.

The first fractions, 1, 2, and 3, eluted by the acetone-water mixture produced no significant inhibition of glucagon. Fraction 4 and especially fraction 5 definitely inhibited glucagon activity. It is apparent from these pre-

TABLE 5. DECREASED HYPERGLYCEMIC ACTION OF GLUCAGON AFTER MIXTURE *in vitro* WITH PAPER-COLUMN CHROMATOGRAM FRACTIONS OF LITHOSPERM

Glucagon	Lithosperm	No. of animals	% increase blood-sugar	% glucagon inhibition
500.0 μgm.	plus 5.0 mg.* fraction 1	4	+68	0
500.0 μgm.	plus 5.0 mg. fraction 2	4	+73	0
75.0 μgm./kg.	control for 1 and 2	4	+67	
500.0 μgm.	plus 5.0 mg. fraction 3	4	+39	− 9.3
500.0 μgm.	plus 5.0 mg. fraction 4	4	+31	−28.0
500.0 μgm.	plus 5.0 mg. fraction 5	4	+14	−67.5
75.0 μgm./kg.	control for 3, 4, and 5	4	+43	

* The dosage of lithosperm is in milligrams of material recovered from the column. Injection volume was about 0.15 ml for the lithosperm-glucagon mixture so that each bird received 75.0 μgm. of glucagon per kilogram of body weight.

liminary results that the fractions which inhibit glucagon are held rather firmly on the column and come off during the elution with water and as the concentration of water increases. The degree of inhibition produced by the 5.0 mg. of fraction 5 was greater than that which followed the administration of the 10.0 mg. eq. of the whole plant extract. It should again be noted that there was no evidence of precipitation of hormone in these lithosperm-glucagon mixtures. Fraction 5, especially, was clear and almost colorless.

The inhibition of glucagon activity following its *in vitro* mixture with lithosperm as reported here parallels closely the results reported for other hormone-lithosperm mixtures. The extensive data for pregnant mare serum "inactivation" by lithosperm appear to us to be very comparable. It seems highly improbable that the lower response of the birds to the glucagon after mixture with lithosperm could be due to *in vivo* inactivating mechanisms. For example, hens which received 2500 mg. of lithosperm powder in five days had blood-sugar levels within the normal range. This is a marked contrast with the mixture of as little as 1.00 mg. of the same powder *in vitro* which produced a 31.2% decrease in the response to glucagon

This occurred in spite of the fact that only 0.15 mg. of the lithosperm was injected (0.15 ml. of the 1.00 mg. lithosperm-glucagon mixture). It is our opinion, therefore, that glucagon is being inactivated by lithosperm when mixed *in vitro*.

It is obvious that additional questions remain to be answered. First, assuming that inactivation does occur, what is the nature of the change in the glucagon molecule? We have been able to demonstrate that crystalline glucagon will be inactivated as well as amorphous glucagon (unpublished data). Furthermore, the active principle of lithosperm has been further fractionated using more refined techniques (unpublished data). We believe, therefore, that we have methods available for attacking this particular problem. Second, we are puzzled by the apparent lack of *in vivo* effect on blood-sugar of the lithosperm. This is especially true since Loeser and his associates have reported such *in vivo* action in rabbits and we have demonstrated that the same powder used in the glucagon experiments inactivates gonadotrophin and thyrotrophin *in vivo* in chicks. Lithosperm, however, is a very complex substance and it is hoped that its further fractionation will assist in resolving this question. Experiments are proceeding using the more purified fractions now available. Liver slices also are being employed for the assay of some of these preparations.

Fortunately, the stability of the lithosperm powder has greatly extended the range of studies of this plant material. Furthermore, the speed with which glucagon activity is inhibited by lithosperm provides a rapid and convenient assay procedure for the various fractions which may be secured from a paper-column or by other means. As was noted in the discussion of Table 4, it is now possible to test for activity in samples of lithosperm of less than 1.00 mg. in amount. Accordingly, a few milligrams of lithosperm powder may now be assayed for inhibitory effect against glucagon, pregnant mare serum, and thyrotrophin.

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LOSS OF ACTH ACTIVITY FOLLOWING INCUBATION OF PITUITARY TISSUE: INHIBITION BY GLUCAGON AND BY PITRESSIN[1]

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ABSTRACT

ACTH activity rapidly disappears from homogenates of anterior lobes of the rat pituitary incubated at 37° C in plasma or in water. Activity disappears relatively slowly from intact anterior lobes incubated in plasma at 37° C. The loss of ACTH activity is probably due to the action of a proteolytic enzyme or enzymes. Destruction is markedly slowed at 5° and at 100° C; the optimum pH for the destructive system is approximately pH 9. Addition of glucagon or Pitressin to the incubates inhibits destruction of ACTH. Plasma proteins and casein appear to have no or negligible protective effect. It has been pointed out that substances added to the medium of incubated pituitaries may increase the quantity of ACTH in the medium by inhibition of ACTH destruction as well as by stimulation of release from adenohypophyseal cells.

ADRENOCORTICOTROPIC hormone (ACTH) activity disappears rapidly from neutral aqueous extracts of pituitary and from neutral aqueous solutions of crude preparations of ACTH when they are allowed to stand at room temperature (1). The demonstration by Bell and co-workers (2, 3, 4) that the hormone is a polypeptide and the observations of Adams and Smith (5) that pituitary tissue contains a variety of proteolytic enzymes are consistent with the idea that proteolysis is in part or wholly responsible for the loss of ACTH activity.

The present study was conducted in order to determine the influence of temperature and of pH on the loss of ACTH activity in incubated pituitary tissue. In addition, it has been demonstrated that glucagon, added to pituitary homogenates, inhibits loss of ACTH activity. This inhibitory action of glucagon was put to experimental test on the basis of the fact that ACTH enhances the glycogenolytic action of glucagon on liver slices and the suggestion of Tyberghein *et al.* (6) that ACTH acts to enhance the *in vitro* activity of glucagon by competing for a degradative enzyme.

Of particular interest to the subject of the mechanism of ACTH release

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is the demonstration reported here that Pitressin inhibits loss of ACTH when added to pituitary homogenates. Saffran and Schally (7) consider the release of ACTH from isolated pituitaries to be the most direct approach to the assay of "corticotropin releasing factor (CRF)." Pitressin has been shown by these workers to increase the ACTH activity of the incubation medium, and has been considered by them to contain CRF as a contaminant of vasopressin. However, the increase in ACTH activity in the medium may be an expression of inhibition of destruction rather than acceleration of release of ACTH from cells.

METHODS

Pituitary glands were obtained from intact and from adrenalectomized male rats, 140 to 250 gm. in body weight. The animals were decapitated, the skull was opened and the pituitary removed with forceps. The neurohypophysis was carefully lifted off and discarded. The adenohypophyses were immediately incubated either intact or as a homogenate. In each case, five anterior lobes were incubated in 2 ml. of distilled water, buffer solution, or plasma.

Incubation of intact anterior lobes. The incubations were carried out in open 10 ml. Erlenmeyer flasks at 37° C in a Dubnoff shaker. Three sets of five glands each were prepared: (1) incubated in 2 ml. of rat plasma, (2) incubated in 2 ml. plasma to which 5 mg. crystalline glucagon had been added, and (3) non-incubated, to which 2 ml. of plasma had been added. The latter served as a control for the determination of the initial concentration of ACTH. At zero time (controls) or at the end of a specified period of incubation, 10 ml. of 0.1N HCl and a few grains of sand were added, the glands ground, and the mixture allowed to stand for 15 minutes. The extract was diluted with 0.01N HCl in 0.9% NaCl in preparation for bioassay. Extraction with 0.1N HCl for 72 hours did not yield more ACTH activity than extraction for 15 minutes.

Incubation of homogenates of anterior lobes. Five anterior lobes were homogenized in each 2 ml. of medium at 0 to 5° C in a Potter-Elvehjem homogenizer and aliquots incubated under various conditions in centrifuge tubes. The tubes were incubated at 37° C without agitation. For a control estimation of the original concentration of ACTH an aliquot from each homogenate was extracted with 0.1N HCl immediately after preparation. At the end of a specified period of incubation 0.1N HCl was added to an aliquot and the extract prepared for bioassay.

Bioassay. The ACTH activity of the 0.01N HCl dilutions was determined by the adrenal ascorbic acid depletion test in hypophysectomized rats (8). Injections were made via the saphenous vein. The final dilutions were prepared in such a way as to make possible the injection of a given fraction of a gland in 0.5 ml. per 100 gm. of assay rat. For example, a "dilution" of five glands to 500 ml. is equivalent to 1/200 of a gland per 0.5 ml.

RESULTS

Incubation of intact anterior lobes

Incubation of anterior lobes for 30 minutes at 37° C resulted in no significant loss in ACTH activity, neither did glucagon increase the concentration of the hormone. Incubation of intact anterior lobes for a period of 8 hours resulted (Table 1) in an estimated 47% loss of ACTH activity in the combined gland plus medium, when compared with the control value.

TABLE 1. CHANGE IN ACTH ACTIVITY IN INTACT ANTERIOR PITUITARY INCUBATED IN PLASMA WITH AND WITHOUT GLUCAGON

Exp. No.	Preparation of anterior pituitary	Dose (fraction of pituitary) per 100 gm. body wt.	Ascorbic acid depletion, mg. per 100 gm. adrenal	
			Individual values	Ave.
1	A. Non-incubated	1/100	125, 199, 157, 116, 136	147
		1/200	71, 80, 33, 56, 83	83
	B. Incubated alone for 30 min.	1/100	128, 157, 164, 186, 122	151
		1/200	67, 86, 80, 50, 25	62
	C. Incubated with glucagon for 30 min.	1/100	125, 201, 141, 146, 134	153
		1/200	66, 39, 96, 62, 47	62
2	A. Non-incubated	1/100	231, 208, 153, 151, 159, 166	178
		1/400	115, 121, 137, 113, 87, 101	112
	B. Incubated alone for 8 hrs.	1/100	248, 203, 150, 72, 131, 143	158
		1/400	93, 56, 57, 68, 46, 38, 44	57
	C. Incubated with glucagon for 8 hrs.	1/100	207, 186, 163, 139, 180	170
		1/400	135, 114, 98, 96, 108, 98, 119	109

Body weight of adrenalectomized rats from which the pituitaries were removed; Exp. 1, 140 to 160 gm.; Exp. 2, 200 to 250 gm. This difference in body weight probably accounts for the difference in ACTH content of pituitaries in the two experiments.

Exp. 1—No significant difference in content of ACTH of pituitaries among groups A, B and C.

Exp. 2—Ratio of content of pituitary ACTH: A/B equals 1.9 (1.5 to 2.4, fiducial limits, one standard error); A/C equals 1.1 (0.9 to 1.4).

Addition of glucagon to the incubation medium completely prevented loss of biological activity.

Incubation of homogenates of anterior lobes

(a) *Rate of inactivation in plasma.* A homogenate was prepared in plasma and incubated at 37° C.; aliquots were withdrawn at various times, extracted and assayed for ACTH activity. Figure 1 is a plot showing the relationship between percentage of ACTH activity remaining and time of incubation. The ACTH activity of the non-incubated control was taken as 100%. There is a considerable loss in activity which appears to follow an exponential curve; only 6% of the original activity remained after 90 minutes of incubation.

(b) *Rate of inactivation in distilled water.* The same experiment was performed using distilled water in place of plasma (Fig. 1). The rate of inactivation in distilled water may be slightly greater than that in plasma. As will be shown subsequently, the slight or negligible inhibitory effect of plasma proteins is in distinct contrast to the marked inhibitory effect of glucagon and Pitressin on the system or systems responsible for loss of ACTH activity.

The above studies were carried out on pituitaries from adrenalectomized rats. The concentration of ACTH in the pituitary three weeks after adre-

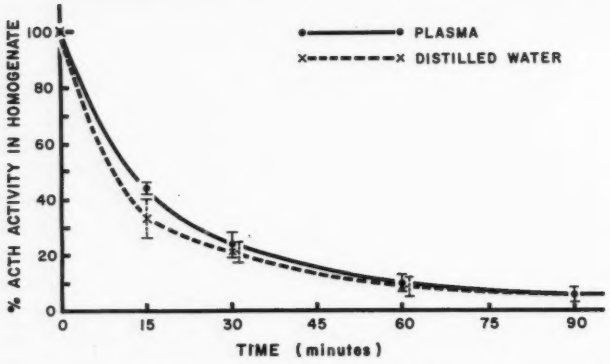

FIG. 1. Percentage reduction in ACTH content of anterior pituitary homogenates with time of incubation at 37° C. Initial content was taken as 100%. The vertical lines define ± one standard error. The estimate for each point was calculated from a multi-dose assay. The doses of the incubated samples were increased to bring the responses into the same order of magnitude as those of the non-incubated controls.

nalectomy is approximately twice that in the pituitary of the intact rat (9). However, an aqueous homogenate of anterior lobes from intact rats exhibited the same relationship between percentage of original activity and time of incubation as presented in Figure 1.

Effect of temperature on incubation

Aliquots of a pituitary homogenate were incubated in distilled water at various temperatures for a 30 minute period. The ascorbic acid depletions evoked by injection of 1/200 gland from each aliquot are shown in Figure 2.

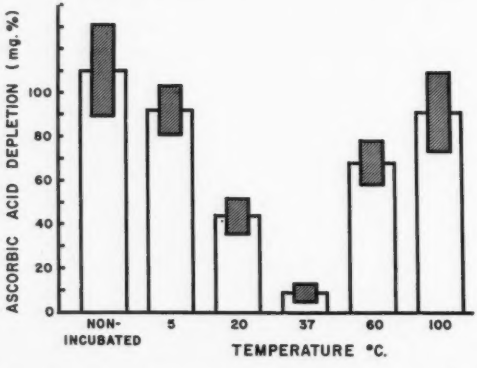

FIG. 2. ACTH activity of anterior pituitary homogenates non-incubated and incubated at various temperatures for 30 minutes. Activity is expressed as adrenal ascorbic acid depletion. Each column represents a mean value for 6 animals and the shaded areas indicate the standard error. Each assay animal received 1/200 lobe per 100 gm. body weight.

TABLE 2. THE EFFECT OF pH ON ACTH DESTRUCTION IN ANTERIOR LOBE HOMOGENATES AT 37° FOR 30 MINUTES

Medium	Dose (fraction of pituitary per 100 gm. body weight)	Ascorbic acid depletion, mg./100 gm. adrenal	
		Individual values	Ave. and S.E.
Control water non-incubated	1/200	64, 92, 76, 72, 66	74 ± 5
0.2 *M* glycine-HCl pH 3	1/200	66, 62, 85, 69, 59	68 ± 5
0.2 *M* Tris* pH 5	1/200	83, 67, 65, 69	71 ± 4
Distilled water	1/50	121, 41, 81, 34	69 ± 20
	1/200	−1, −29, 5, 13	− 3 ± 6
0.2 *M* phosphate pH 7	1/50	107, 39, 51, 72	67 ± 12
	1/200	−21, −45, 17, 30	− 5 ± 12
0.2 *M* glycine NaOH pH 9	1/50	12, 12, −60, 21, 20	1 ± 14
	1/200	−19, −7, −14, −25	−16 ± 5
0.2 *M* $NaHCO_3$-Na_2CO_3 pH 11	1/200	74, 67, 60, 70	68 ± 3

Body weight of rats which donated pituitaries, 140 to 160 gm.
* Tris (hydroxymethyl) aminomethane.

It can be seen that at 37° C complete destruction of activity was demonstrable, whereas at 5° C and 100° C there was no appreciable loss of ACTH activity.

Effect of pH on incubation

Aliquots of an anterior lobe homogenate were incubated in various buffer solutions (10) at 37° C for 30 minutes. The results are presented in Table 2. There was no significant difference between the responses obtained following doses of 1/200 lobe from the non-incubated control sample and the samples incubated at pH 3, 5 and 11. In contrast, the same doses from aliquots incubated at pH 7 and 9 were inactive. However, when four times the dose (i.e., 1/50 lobe) of these samples was given significant activity was demonstrated in the instance of pH 7, but not in the instance of pH 9. These findings suggest that maximal inactivation occurs in the vicinity of pH 9.

Effect of additions to the homogenate

The addition of 1 mg. of glucagon per gland to the homogenate was shown to be equally effective as in the intact gland experiment in protecting ACTH against destruction during incubation. This quantity of glucagon alone did not produce ascorbic acid depletion in the hypophysectomized rat. The addition of smaller, graded doses of glucagon to aliquots of a pituitary homogenate resulted in various degrees of protection of ACTH activity (Fig. 3). Glucagon at a concentration of 100 μg. per gland appeared to give complete protection.

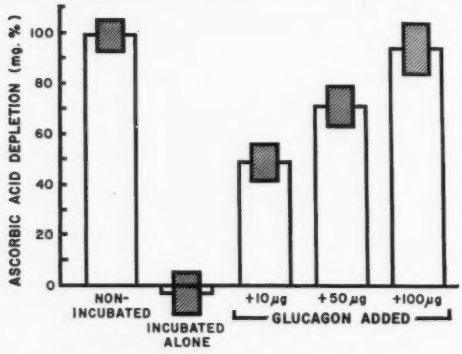

FIG. 3. ACTH activity of anterior pituitary homogenates non-incubated and incubated at 37° C for 30 minutes with and without glucagon. The activity is expressed as adrenal ascorbic acid depletion. Each column represents a mean value for 8 animals and the shaded areas indicate the standard error. Each assay animal received 1/200 lobe per 100 gm. body weight.

One mg. or smaller graded doses of Pitressin (Parke, Davis, lot No. 168941) also exerts an inhibitory effect on the system or systems responsible for loss of ACTH activity (FIG. 4). When Pitressin was injected into hypophysectomized rats in the quantity present in the strongest final dilution (0.033 unit per 0.5 ml.) there was no significant ascorbic acid depletion. Furthermore, the addition of 100 μg. of Pitressin to a pituitary homogenate immediately before injection into a hypophysectomized test rat did not enhance the activity of the ACTH present in the homogenate. The changes observed in the incubated homogenates following addition of Pitressin appear therefore to be due to inhibition of destruction of ACTH.

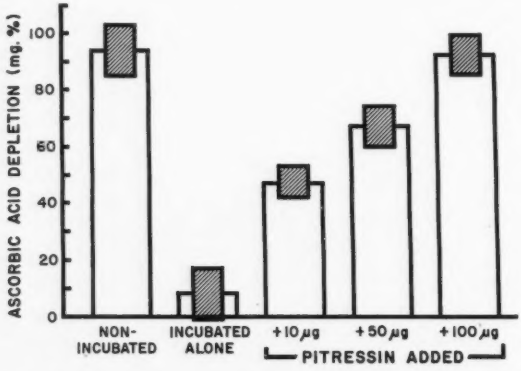

FIG. 4. ACTH activity of anterior pituitary homogenates non-incubated and incubated at 37° C for 30 minutes with and without Pitressin. The activity is expressed as adrenal ascorbic acid depletion. Each column represents a mean value for 8 animals and the shaded areas indicate the standard error. Each assay animal received 1/200 lobe per 100 gm. body weight.

The addition of casein (Merck) to a homogenate in a concentration of 2.5 mg. per gland did not appear to exert any protective action on the biological activity of ACTH. This quantity, 2.5 mg. of casein, is of the same order of molar concentration as that of the effective concentration of glucagon.

DISCUSSION

The experiments demonstrate the presence in adenohypophysis of a system or systems which rapidly inactivate ACTH activity. The temperature and pH dependence of the system suggests that it is enzymatic in nature. Ghosh *et al.* (1) have already presented evidence to support this hypothesis. That a proteolytic enzyme or enzymes is involved is suggested by the following facts: (1) ACTH is a polypeptide (2, 3, 4), (2) highly purified preparations of the hormone lose activity following hydrolysis by proteolytic enzymes (3, 4) and (3) various exopeptidases and at least two proteinases are present in hog and beef pituitaries (5). Definitive characterization of the enzyme(s) involved in ACTH destruction must await isolation in purified form from pituitary tissue.

Biological activity of ACTH is lost when the hormone is incubated with tissue other than pituitary. Geschwind and Li (11) have demonstrated that homogenates of liver, kidney, and adrenal will destroy the activity of added preparations of ACTH.

The relatively slow loss of ACTH activity following incubation of intact anterior lobes of pituitary as compared to homogenates of the tissue supports the suggestion of Ghosh *et al.* (1) that ". . . ACTH and enzymes which destroy ACTH occupy different loci in the adenohypophyseal cell." Of interest in this connection is the demonstration by Gianetto and De Duve (12) that cathepsin of liver is bound to a special cytoplasmic granule, the lysosome. Other possible mechanisms to explain the phenomenon should be considered, for example, an inhibitor of the ACTH-inactivating system may protect biological activity in the intact cell. Disruption of cell architecture may remove this inhibitor or negate its action; ACTH destruction in the homogenate would represent the relief of an inhibitory action on the ACTH-inactivating system.

It is doubtful that the system present in the adenohypophysis concerned with the inactivation of ACTH plays a physiological role. The fact that inactivation goes on at a slow rate in intact cells suggests that the degradative system is part of a proteolytic complex concerned with the hydrolysis of protein to peptides and amino acids subsequent to cell death.

The glycogenolytic activity of glucagon is dramatically increased by the simultaneous addition of ACTH or insulin to the liver slice (6). Added polypeptides are presumed to act by substrate competition in decreasing the rate of destruction of glucagon by tissue proteolytic enzymes. Glucagon and Pitressin may be acting by a similar mechanism in protecting ACTH activity in incubated pituitary. However, the exact nature of the

protective action, competitive inhibition, or non-competitive inhibition, remains to be established. This would involve isolation and characterization of the enzyme(s) concerned with the destruction of ACTH activity. Furthermore, it would be of interest to establish whether ACTH, glucagon and Pitressin are substrates of the same enzyme system.

Plasma proteins and casein, in contrast to glucagon and Pitressin, exert no, or a negligible inhibitory influence upon the ACTH inactivating system. This suggests that the inhibitory effect is confined to polypeptides. It is of interest in this connection that certain substances increase the quantity of ACTH in the medium of incubated pituitary. This increase has been ascribed to the release of ACTH from isolated pituitary and the *in vitro* system has been employed as an assay for the corticotropin releasing factor (CRF) (7, 13, 14). The results of the present study justify expression of a note of caution in the acceptance of increase in ACTH in the medium as a measure of CRF activity. The added substance may act to inhibit destruction of ACTH; increased quantities of activity in the medium may be at least in part a consequence of inhibition of destruction rather than stimulation of release of ACTH.[3]

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[3] The routine experimental conditions employed by Saffran and Schally (7) differ in certain important details from those employed in the present study. Saffran and Schally cut the anterior lobe into two parts; whole lobes were incubated in the present study. Saffran and Schally analyzed the medium; the combination, tissue plus medium, was analyzed in the present study. For these reasons no direct comparisons of the two studies can be made. However, the action of additives in the experiments of Saffran and Schally may be explained as due to inhibition of destruction of ACTH in the medium as well as by stimulation of ACTH release from the pituitary. The cut surface of the gland may be expected to release proteolytic enzymes in addition to ACTH into the medium and progressive destruction of ACTH by the proteolytic enzymes would be anticipated. The addition of "CRF" to the medium may inhibit ACTH destruction and thus lead to an increase in the final ACTH content of the medium as compared to preparations to which no "CRF" had been added. The conclusion that more ACTH had been released from the pituitary would be erroneous.

An important consideration is whether the amount of material added to the medium in the experiments of Saffran and Schally is enough to act as an inhibitor of proteolysis. Saffran and Schally estimate the quantity of ACTH to be 15 milliunits in the control media and 30 to 45 milliunits in the media to which "CRF" had been added as determined by their corticosteroid assay. These amounts correspond to 5 and 10 to 15 milliunits by the adrenal ascorbic acid depletion test, respectively. Schally and Saffran (15) report that 0.1 microgram of a highly purified "CRF" was active in "releasing" ACTH into the medium of incubated pituitaries. The ratio of this quantity of "CRF" to ACTH (0.1 microgram to 10 milliunits of ACTH) is of the same order of magnitude as the ratio of the quantity of glucagon to ACTH in the present experiments (10 micrograms of glucagon to 500 milliunits of ACTH).

Experiments may be designed to settle the question. Medium separated from halved pituitaries after 10 minutes of incubation may be divided into 2 aliquots. To one aliquot add "CRF" or glucagon and to the other make no addition. Incubate both samples for one hour. Equal quantities of ACTH in the control and test samples would support the "release" concept; less ACTH in the control than in the sample to which "CRF" had been added would support the "inhibition of destruction" concept.

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THYROID I^{131} METABOLISM IN THE ABSENCE OF THE PITUITARY: THE UNTREATED, HYPOPHYSECTOMIZED RAT[1]

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ABSTRACT

Hypophysectomized rats and their controls were injected with 5 μg. of iodide labeled with I^{131}. At intervals ranging from 45 minutes to 8 days, measurements were made of the total thyroid uptake of I^{131} and of the chemical distribution of I^{131} in the thyroid gland and in the plasma. T/S ratios were also measured. Total thyroid uptake of I^{131} was reduced by hypophysectomy to only a few per cent of control values, and T/S ratios were also greatly reduced. Previous iodine intake, however, exerted a marked influence on the values obtained. Thyroglobulin formation in the absence of the pituitary was reduced to very low levels. The rate was estimated in two experiments to be about one per cent that of the controls. Thyroglobulin that was formed by the thyroids of hypophysectomized rats, moreover, contained only very low percentages of I^{131}-thyroxine and other iodothyronines, but a normal percentage of I^{131} diiodotyrosine and a greater than normal fraction of I^{131}-monoiodotyrosine. The plasma of hypophysectomized rats contained no detectable amounts of I^{131}-thyroxine, even as late as 8 days after I^{131} injection. The striking reduction in all phases of thyroid iodine metabolism observed in rats without pituitaries leads us to emphasize the importance of pituitary, rather than autonomous, control in the regulation of thyroid iodine metabolism.

IN 1942, Morton and Chaikoff and their coworkers (1) reported that the formation of diiodotyrosine in the thyroid gland of the rat was not interfered with by hypophysectomy, but that thyroxine formation, on the other hand, was greatly depressed. They postulated that removal of the pituitary in rats imposed a specific block on the conversion of diiodotyrosine to thyroxine by the thyroid.

These results were questioned by Albert and Lorenz (2), and by Roche and Michel and their coworkers (3). Both groups reported that significant I^{131}-thyroxine formation occurred in the thyroids of hypophysectomized rats although the rates were below those observed in control animals. They concluded that removal of the pituitary decreased the over-all conversion of iodide to thyroxine, but that no specific block occurred on the conversion of diiodotyrosine to thyroxine.

In order to clarify the discrepancy between the early findings in this

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laboratory and those of subsequent workers, we have reinvestigated thyroid iodine metabolism in hypophysectomized rats, making use of the improved techniques now available for the hydrolysis of thyroglobulin and for the separation of iodinated amino acids. In the present study, we confirmed the finding of Morton *et al.* (1) with respect to the striking depression of I^{131}-thyroxine formation by the thyroids of hypophysectomized rats. However, in agreement with later investigators, we found that other phases of thyroid iodine metabolism are also greatly reduced by hypophysectomy.

METHODS

Animals.—Hypophysectomized and normal control rats were purchased locally.[2] Most of them were Long-Evans males, weighing approximately 200 gm. at the time of surgery. In one experiment, Sprague-Dawley rats of similar weight were used.

Hypophysectomy was performed by a skilled tchnician trained in the Institute of Experimental Biology in this University. The pituitary gland was approached by the parapharyngeal route, and a dental drill and gentle suction were employed in removing the gland. At autopsy, the heads of the rats were checked with a dissecting microscope for the presence of pituitary tissue. Only three animals were rejected on the basis of incomplete hypophysectomy, out of a total of more than 100 operated rats used in this investigation.

Diet.—The animals were raised on a diet consisting of Purina Laboratory Chow, supplemented every fourth day with chicken scratch and every seventh day with whole wheat kernels. This diet was relatively rich in iodine, as shown by analysis of one batch (6.5 μg. iodine per gm.) and also as indicated by low thyroidal I^{131} uptakes in rats maintained on it. Since the iodine content of the diet was thought to be an important factor in experiments of the type reported here, it was decided to use groups of rats with differing iodine intakes. The dietary treatment of the rats varied as follows:

Group A.—These rats, on arrival in our laboratory one or two days after hypophysectomy, were placed on a low iodine diet of the following composition: 67.5% whole wheat, 15% casein, 10% whole milk powder, 1% NaCl, 1.5% chalk, 5% hydrogenated vegetable oil, and 50 ml. of a vitamin A- and D-containing fish oil per 8 kg. of diet. Several batches of this diet were prepared in the course of this investigation. Iodine analyses were performed on only two of these batches, yielding in one case 0.2, and in the other 0.4 μg. of iodine per gm.

Group B.—These rats were fed the above low iodine diet for two or five weeks *before* removal of the pituitary. The same diet was also fed after hypophysectomy.

Group C.—These rats were maintained on the Purina-base diet both before and after hypophysectomy.

In all three groups, the diet fed to the hypophysectomized rats was also fed to their respective controls. The average daily food intake of hypophysectomized rats varied between 7 and 18 gm.; most of them ingested 10–15 gm. per day.

Control rats were not allowed to eat *ad lib.* An attempt was made in the early experiments to pair-feed them with the operated rats, but this proved impractical. In later experiments, the food intake of each control rat was restricted to 15 gm. per day.

I^{131} *for injection.*—Oak Ridge I^{131} was used without further purification. Hypophysectomized rats were injected intraperitoneally with approximately 500 μc. of I^{131}, whereas controls received 25–50 μc. All injections included 5 μg. of iodide carrier. Iodide

[2] Diablo Animal Laboratories, Berkeley, California.

carrier was used for two reasons: 1) to bring the concentration of circulating iodide in normal and hypophysectomized rats up to approximately the same level, at least during a short interval after the injection; and 2) to accentuate possible differences in capacity between the thyroids of normal and operated rats. Five μg. of iodide carrier was selected as a suitable dose in the physiological range.

Interval between hypophysectomy and sacrifice.—The rats were injected with I^{131} 7–10 days after hypophysectomy. Groups of animals were sacrificed at intervals ranging, in most instances, from 45 minutes to 77 hours after I^{131} injection. In one experiment, the intervals between I^{131} injection and sacrifice ranged up to 8 days.

Chromatographic analysis of thyroid tissue.—The rats were anesthetized with Nembutal, and their thyroids were removed with the aid of a dissecting microscope and weighed on an analytical balance. In the early stages of this investigation, thyroid glands from two rats were generally pooled for chromatographic analysis, but in later experiments, the procedure was modified to permit analyses on the thyroids of a single rat. In the former case, the tissue was homogenized with a dry, conical, motor-driven, glass homogenizer at 0° C, with 0.50 ml. of Krebs-Ringer-bicarbonate buffer containing 0.001 *M* thiouracil. In the latter case, the homogenization procedure was the same, except that 400 μl. of the bicarbonate buffer were used. Twenty μl. of the homogenate were chromatographed on filter paper in collidine-water-NH_3, as previously described (4, 5), for determination of the I^{131} compounds in the unhydrolyzed thyroid. After removal of this aliquot, pancreatin (Merck) was added (20 mg./ml.), and the homogenate was digested at 37° for 24 hours under toluene. After this interval, additional pancreatin was added (10 mg./ml.), and the incubation was continued for another 24 hours. Twenty μl. of the thyroid digest were then chromatographed in collidine-water-NH_3. Radioautograms were prepared from the dried paper chromatograms, as previously described (4). For quantitative estimation of the I^{131} in the various components, sections of the chromatograms corresponding to the bands observed on the radioautograms were cut out, folded, and placed in vials for counting in a well-type scintillation counter. The counter had a sensitivity of 6×10^5 counts per minute per μc. of I^{131}, and a background of 80–90 counts per minute.

Determination of T/S ratios in the presence of propylthiouracil.—Two different procedures were employed: 1) *Group A*—The rats were injected first with 1 ml. of propylthiouracil solution (20 mg./ml.), and 30 minutes later with I^{131} containing 5 μg. of iodide carrier. At intervals of 75 minutes, 4 hours, and 8 hours thereafter, two rats were sacrificed. The rats in the 8-hour group received a second injection of 10 mg. of PTU approximately four hours after the first injection. The thyroids were weighed and homogenized at 0° with 0.50 ml. of Krebs-Ringer-bicarbonate solution in a dry, conical homogenizing tube. Twenty μl. of the homogenate were chromatographed in collidine-water-NH_3. The inorganic section of the chromatogram was cut out and counted, and at the same time a small aliquot of plasma from the same rat was counted. The inhibition of organic iodine formation was complete in all cases, as shown by the radioautograms. The T/S ratio was calculated from the formula:

$$\frac{\text{Iodide-}I^{131}\text{ on chromatogram} \times 25 \times 1000}{I^{131}\text{ per ml. of plasma} \times \text{mg. of thyroid tissue}}.$$

2) *Groups B and C.*—These rats were injected with 1 ml. of propylthiouracil solution (20 mg./ml.), and 30 to 60 minutes later with I^{131} containing 5 μg. of iodide carrier. The animals were sacrificed 70–75 minutes after receiving I^{131}, and their thyroids were weighed and then counted *in toto* in the scintillation counter. Inhibition of organic iodine formation was assumed to be complete in all cases, and the T/S ratio was calculated from the formula:

$$\frac{\text{Total } I^{131}\text{ per gm. of thyroid tissue}}{\text{Total } I^{131}\text{ per ml. of plasma}}.$$

After they had been assayed for I^{131}, the thyroids were transferred to digestion flasks, and the I^{127} content was determined by a method previously described (6).

Determination of T/S ratios in unblocked thyroids.—The determination of T/S ratios in unblocked thyroids requires a procedure for estimating thyroid inorganic I^{131} in the presence of a great excess of thyroglobulin I^{131}. In a previous investigation (7), we estimated the inorganic I^{131} content of thyroids from filter paper chromatograms of the unhydrolyzed homogenates. As pointed out by Wollman and Scow (8), however, the values for inorganic I^{131} obtained by this procedure may be too high because a small breakdown of thyroglobulin, which usually comprises over 95% of the total thyroid I^{131}, may occur at some stage of the chromatographic procedure. To avoid this objection, inorganic-I^{131} was determined by chromatographing an ethanol extract of the unhydrolyzed thyroid homogenate. Such an extract contained very little of the gland's thyroglobulin, and control experiments showed that it removed quantitatively inorganic-I^{131} added to thyroid tissue. The details of the procedure were as follows: 100 μl. of the unhydrolyzed homogenate were added to 200 μl. of cold, 95% ethanol. The mixture was centrifuged at 0° C, and 60 μl. of the clear supernatant were transferred to a filter paper strip (in three 20-μl. portions) for chromatography. The inorganic I^{131} section of the chromatogram was cut out and counted as described above. The values obtained with this procedure were usually much lower then those obtained from chromatograms of the untreated homogenate.

The above procedure for inorganic I^{131} was applied to the thyroids of some of the rats in each group. The T/S ratio of the unblocked thyroid was then obtained from the formula:

$$\frac{\text{Inorganic } I^{131} \text{ per gm. of thyroid}}{\text{Inorganic } I^{131} \text{ per ml. of plasma}}.$$

RESULTS

I. *Total Uptake of I^{131} by the Thyroids of Hypophysectomized Rats*

As shown in Figure 1, hypophysectomy greatly reduced the uptake of I^{131} by the thyroids of all the rats studied here. Actual uptake values, however, were greatly affected by previous dietary treatment. The thyroids of the rats of Group B, for example, showed the highest uptake values, both in the controls and in the operated animals. The peak uptake in the controls was approximately 30% (12–24 hours), whereas the uptakes in the corresponding operated rats reached an average value of 1.8% in 77 hours. On the other hand, the thyroids of the rats of Group C showed the lowest uptake values—approximately 3% for the controls and 0.16% for the hypophysectomized animals. The hypophysectomized rats in Group A showed thyroidal I^{131} uptakes somewhat higher than those of Group C. These findings indicate that the uptake of I^{131} by the thyroids of rats without pituitaries may be significantly increased by administering a low iodine diet before hypophysectomy. The high uptake values reported for hypophysectomized rats by Morton *et al.* (1) and by Albert *et al.* (2) probably also may be attributed, at least in part, to the feeding of a low iodine diet before removal of the pituitary.

II. *Distribution of I^{131} in Thyroids and Plasma*

Unhydrolyzed thyroids.—The curves in Figure 2 illustrate the distribu-

tion of I^{131} in the unhydrolyzed thyroids of the rats of Groups A and B. In both normal and hypophysectomized rats, at all intervals from 45 minutes to 72 hours after I^{131} injection, thyroglobulin and iodide accounted for practically all of the I^{131} in the thyroid gland. Small amounts of free I^{131}-diiodotyrosine were noted in control thyroids only at later intervals, and at no time up to 72 hours were more than traces of free I^{131}-thyroxine observed.

As shown in Figure 2, the I^{131} taken up by the thyroids of hypophysec-

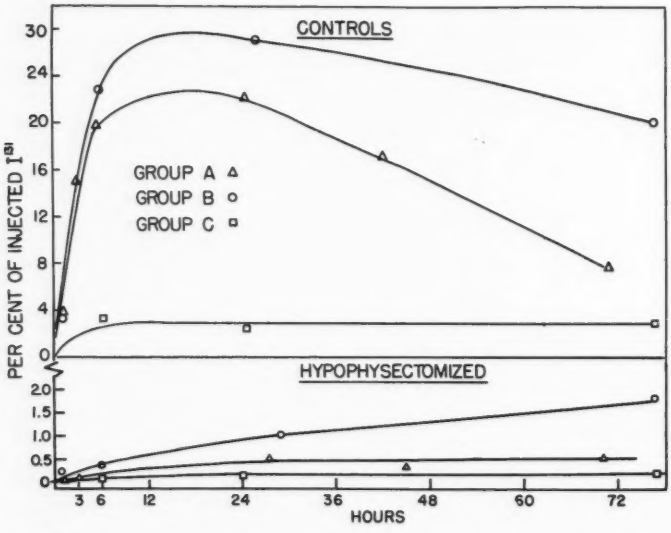

FIG. 1. Thyroid uptake of I^{131} in hypophysectomized and control rats injected with 5 μg. of labeled iodide. *Group A:* Placed on low iodine diet *after* hypophysectomy. *Group B:* Placed on low iodine diet 5 weeks before hypophysectomy. *Group C:* Maintained on Purina-base diet throughout. See text for details. Each point represents the average of results obtained with 2 or 3 rats.

tomized rats was not so rapidly converted to thyroglobulin as was the I^{131} accumulated by control thyroids. After 45 minutes, for example, 45–50% of the I^{131} in the thyroids of the operated rats was still inorganic, whereas the corresponding figure in control rats was usually less than 5%. At the end of 6 hours, however, the distribution of I^{131} in the unhydrolyzed thyroids of hypophysectomized rats was almost indistinguishable from that of controls.

The results obtained for the rats in Group C are not plotted in Figure 2 because no data were obtained for this group at early intervals. However, the data which are available (6 hours and thereafter) agree essentially with the results shown in Figure 2.

The values for inorganic iodide shown in Figure 2 were obtained from chromatograms of the untreated thyroid homogenate. For reasons outlined under "Methods," these values are probably too high.

From the measurements made at the 45-minute interval, it is possible to calculate the micrograms of injected iodide (I^{127}) converted to thyroglobulin by the thyroids of hypophysectomized and control rats (on the likely assumption that none of the newly synthesized thyroglobulin left

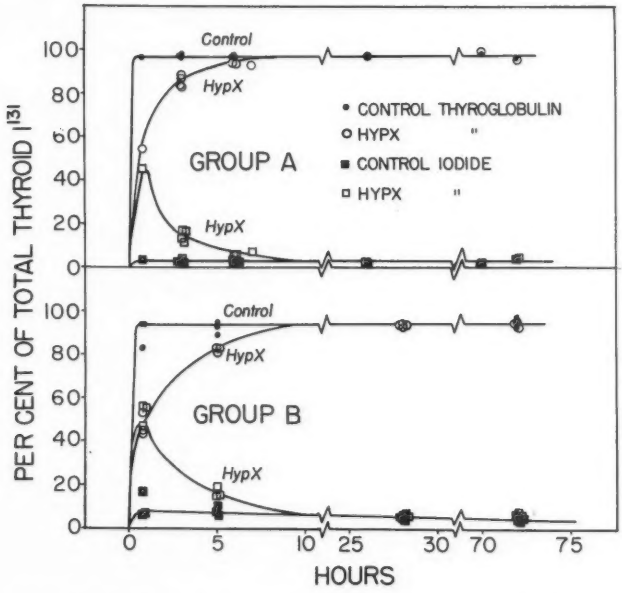

FIG. 2. Distribution of I^{131} in *unhydrolyzed* thyroids of normal and hypophysectomized rats at intervals after the injection of 5 μg. of labeled iodide. *Group A:* Placed on low iodine after hypophysectomy. *Group B*: Placed on low iodine diet 2 weeks *before* hypophysectomy. Each point in Group A represents a result obtained with the pooled thyroid of 2 rats. Each point in Group B represents the thyroids of a single rat. HypX = hypophysectomized.

the gland during this interval). Such values, shown in Table 1, were obtained by multiplying 5 μg. (the injected dose) by a factor representing the product of 1) the thyroidal uptake of I^{131} and 2) the fraction of the total I^{131} present as thyroglobulin. From these data it appears that the thyroids of control rats converted *injected* iodide to thyroglobulin at a rate of 0.10–0.2 μg. per hour, whereas the corresponding value for hypophysectomized rats was only 0.001 μg. per hour. Several important factors must be considered in determining the extent to which these values reflect not simply the conversion of injected iodide, but the relative conversion of *total* iodide to thyroglobulin. These are dealt with below.

TABLE 1. CONVERSION OF INJECTED I^{127} TO THYROGLOBULIN BY THYROIDS OF HYPOPHYSECTOMIZED AND CONTROL RATS INJECTED WITH 5 μg. OF LABELED IODINE

Group	Average per cent uptake of I^{131} by thyroids in 45 min.		Average per cent of thyroid I^{131} in thyroglobulin after 45 min.		Micrograms of injected I^{127} converted to thyroglobulin in 45 min.	
	Control	HypX	Control	HypX	Control	HypX
	per cent	per cent	per cent	per cent	μg.	μg.
A (2)	3.6	0.034	96	55	0.17	0.00094
B* (3)	1.8	0.39	90	47	0.085	0.00092

Figures in parentheses refer to number of rats in group.
* Low iodine diet fed for 2 weeks before hypophysectomy.

Hydrolyzed thyroids.—Typical radioautograms showing the distribution of I^{131} in enzymatically hydrolyzed thyroids of hypophysectomized and control rats are shown in Figure 3. Inspection of these results, obtained with thyroids removed 77 hours after I^{131} injection, reveals clearly that I^{131}

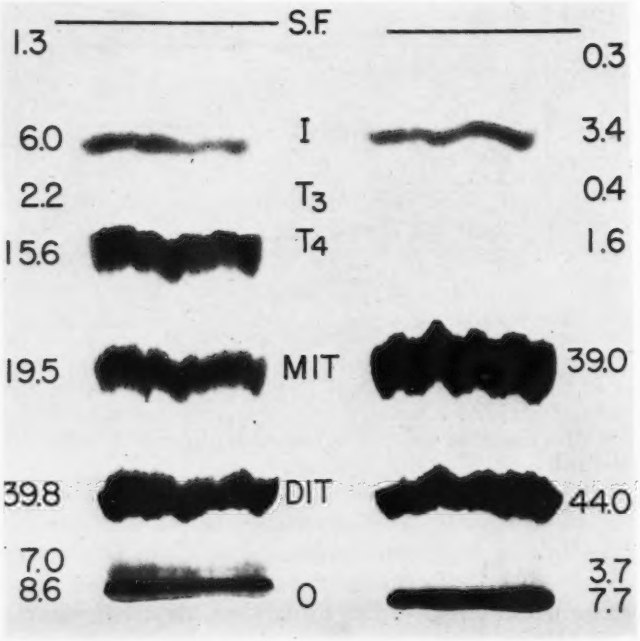

FIG. 3. Radioautographs of filter paper chromatograms showing the distribution of I^{131} in pancreatin digest of thyroids 77 hours after I^{131} injection. Left, control rat. Right, hypophysectomized rat. Explanation of symbols: O, origin; DIT, diiodotyrosine; MIT, monoiodotyrosine; T_4, thyroxine; T_3, 3′,3,5, triiodothyronine; I, inorganic iodide; S.F. solvent front. The numbers refer to the percentage of the I^{131} on the chromatogram present in various components. Solvent, collidine-water-NH_3.

which is taken up by the thyroids of hypophysectomized rats is readily converted to mono- and diiodotyrosine, but not to thyroxine. Similar radio-autograms were obtained with thyroids removed at intervals ranging from 45 minutes to 8 days after I^{131} injection.

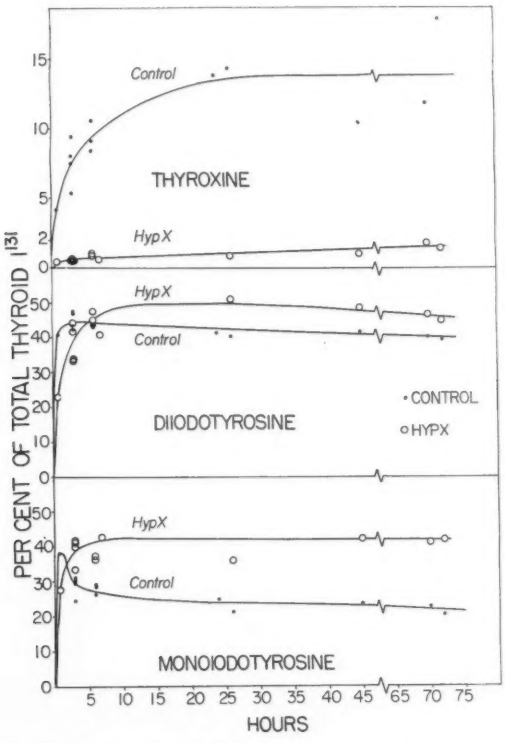

FIG. 4. Distribution of I^{131} in enzymatic digests of thyroids from hypophysectomized and control rats of Group A. These animals were placed on a low iodine diet after removal of the pituitary. Each point represents a result obtained with the pooled thyroids of 2 rats. HypX = hypophysectomized.

The line drawings in Figures 4–6 summarize the chromatographic findings obtained for the different dietary groups. The points for these curves were obtained by radioactive assay of paper chromatograms corresponding to radioautograms such as those illustrated in Figure 3. The findings were similar in all groups, and may be summarized as follows: 1) The thyroglobulin formed by the thyroid gland in the absence of the pituitary contains relatively little I^{131}-thyroxine. Even as late as 196 hours after injection of I^{131}, the I^{131}-thyroxine in the thyroids of rats without pituitaries amounted to about 2.5% of the total I^{131}, compared with a control value

of almost 20%. 2) The I^{131}-diiodotyrosine content of the thyroglobulin formed by the thyroids of hypophysectomized rats was not much different from that in controls. The I^{131}-monoiodotyrosine content, however, was significantly higher.

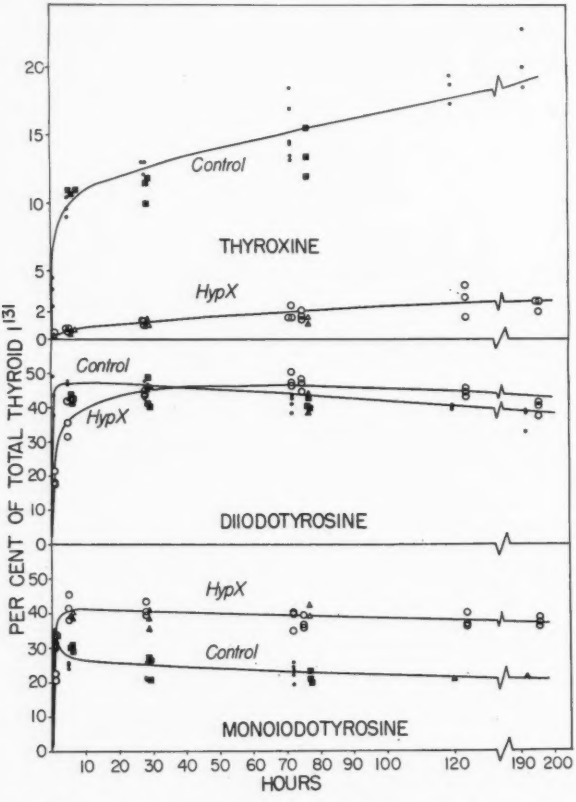

FIG. 5. Distribution of I^{131} in enzymatic digest of thyroids from hypophysectomized and control rats of Group B. These rats were placed on a low iodine diet 2 or 5 weeks *before* removal of the pituitary. Each point represents results obtained with the thyroids of a single rat.

- • Controls on low iodine diet 2 weeks before hypophysectomy.
- ■ Controls on low iodine diet 5 weeks before hypophysectomy.
- ○ = Hypophysectomized on low iodine 2 weeks before hypophysectomy.
- △ Hypophysectomized on low iodine 5 weeks before hypophysectomy.

HypX = hypophysectomized.

Our results indicate, therefore, not only that thyroglobulin formation by the thyroid gland is reduced to very low levels after hypophysectomy, but also that the thyroglobulin which is formed does not have the normal distribution of iodinated amino acids. Thyroglobulin, newly synthesized

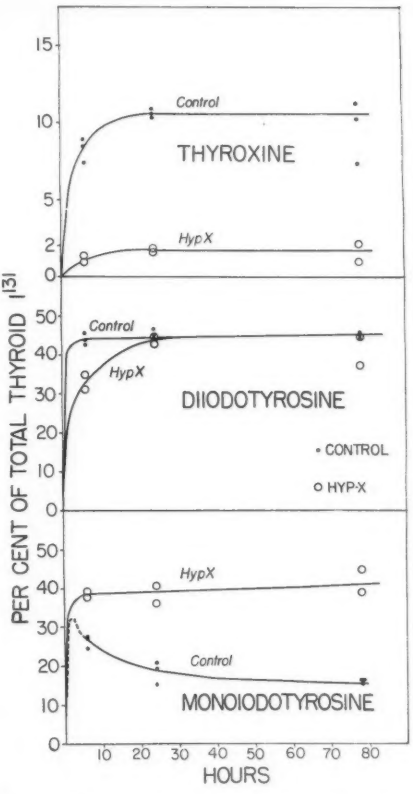

FIG. 6. Distribution of I^{131} in enzymatic digest of thyroids from hypophysectomized and control rats of Group C. These rats were fed the Purina-base diet both before and after hypophysectomy. Each point represents a result obtained with the thyroids of a single rat. HypX = hypophysectomized.

in the absence of the pituitary, appears to have a greatly reduced thyroxine content and a somewhat elevated monoiodotyrosine content. The absence of the pituitary, therefore, seems to affect the formation of thyroxine by rat thyroids to a much greater extent than it affects the formation of iodotyrosines.

Plasma.—In agreement with previous observations (1, 2), it was found here (Figure 7) that injected I^{131}-iodide disappears more slowly from the plasma of hypophysectomized rats than from the plasma of control rats. This may be attributed to two factors: 1) the decreased uptake of I^{131} by the thyroids in the absence of the pituitary, and 2) the decreased renal clearance of inorganic iodide (9).

The appearance of I^{131}-thyroxine in the plasma of normal and hypophysectomized rats is also shown in Figure 7. I^{131}-thyroxine was readily detectable in the plasma of control rats as early as 6 hours after injection of I^{131}-iodide, but in rats without pituitaries the chromatograms of plasma showed only negligible amounts of labeled thyroxine at all intervals up to 8 days. The I^{131}-thyroxine values shown for hypophysectomized rats in Figure 7 (0.02% of the injected dose per 100 ml. of plasma) are based on such low-counting rates that they are of questionable significance.

III. *The Iodide-concentrating Capacity of Thyroids of Hypophysectomized Rats*

Previous workers have established that the iodide-concentrating capacity of the rat thyroid, as measured by the T/S ratio, is greatly reduced after hypophysectomy (8, 10, 11). It seemed of interest to carry out similar measurements on the hypophysectomized rats used in this study. Our

results are shown in Tables 2 and 3. Results with both blocked and unblocked thyroids are presented for rats in the different dietary groups. Thyroid iodine (I^{127}) analyses are also shown for many of the rats.

Thyroids blocked with propylthiouracil.—Hypophysectomy greatly reduced the T/S ratios in all the rats studied here (Table 2). The absolute values, however, varied greatly depending on previous dietary treatment. The results may be summarized as follows: *Group C*—Hypophysectomized rats which had been raised on the Purina-base diet and which were fed the same diet after hypophysectomy had very low T/S ratios, averaging ap-

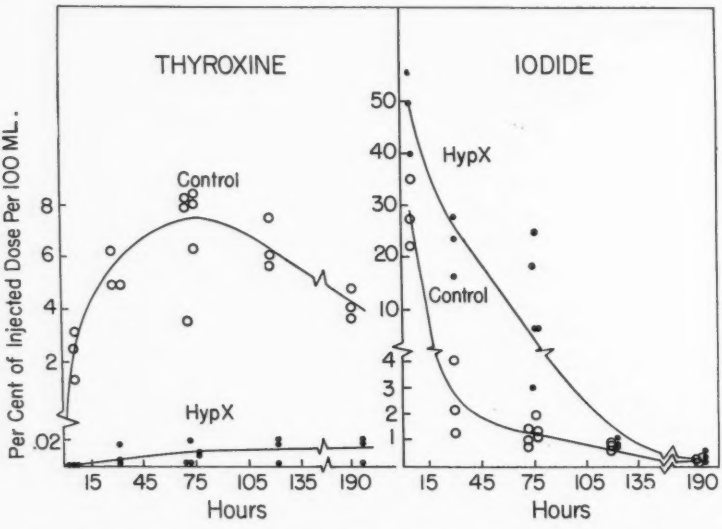

FIG. 7. Left, appearance of I^{131}-thyroxine in plasma of control and hypophysectomized rats of Group B (5 weeks on low iodine diet before hypophysectomy). Right, disappearance of I^{131}-iodide from plasma of same animals.

proximately 2. Control T/S ratios in this group averaged approximately 25. *Group A*—The rats in this group were transferred to the low iodine diet *after* hypophysectomy. Their T/S ratios, and those of their controls, were determined after the low iodine diet had been fed for 8–10 days. Under these conditions, the control T/S ratios averaged more than 100, but the values for the hypophysectomized rats were all in the range 1.6–3.2, not significantly different from those of hypophysectomized rats fed the high iodine diet. *Group B*—The feeding of a low iodine diet for 2 or 5 weeks *before* hypophysectomy greatly increased the T/S ratios in both the control and hypophysectomized animals. Control values averaged 250 in the group fed the low iodine diet for 5 weeks, compared with a value of 24 for the hypophysectomized rats.

It is apparent from these results that T/S ratios in hypophysectomized rats are profoundly affected by the diet fed to the animals before hypophysectomy. The T/S ratios of hypophysectomized rats exposed to a low iodine diet for several weeks before hypophysectomy (Group B) were equally as high as those of *control* rats that had been fed a diet relatively high in iodine (Group C). On the other hand, the feeding of a low iodine diet to hypophysectomized rats after hypophysectomy (Group A) had no effect on the T/S ratio in our experiments, although Halmi *et al.* previously

TABLE 2. T/S RATIOS AND THYROID I^{127} CONCENTRATIONS IN HYPOPHYSECTOMIZED AND CONTROL RATS TREATED WITH A SINGLE BLOCKING DOSE OF PROPYLTHIOURACIL

Group	Interval after I^{131}	Hypophysectomized			Control		
		Thyroid weight	T/S	Thyroid I^{127} conc.	Thyroid weight	T/S	Thyroid I^{127} conc.
	hours	mg.		mg. %	mg.		mg. %
A							
Rats placed on low iodine diet after hypophysectomy	1.25	11.7	2.9		13.2	96	
	1.25	8.5	1.7		14.8	89	
	4	10.9	3.2		13.7	111	
	4	9.6	1.8		14.5	121	
	8	9.0	1.9		15.2	137	
	8	10.5	1.6		15.8	150	
B							
Rats placed on low iodine diet 2 weeks before hypophysectomy	1.15	8.2	15	93	13.0	156	77
	1.15	6.9	21	79	10.8	75	57
	1.15	10.5	4.7	147	10.6	116	87
					7.8	140	84
Rats placed on low iodine diet 5 weeks before hypophysectomy	1.15	8.0	23	101	13.1	220	58
	1.15	7.4	36	81	11.5	342	58
	1.15	9.0	29	72	13.7	277	61
	1.15	12.2	12	75	11.3	268	50
	1.15	10.1	20	82	8.3	154	49
C							
Rats maintained on Purine-base diet throughout	1.25	8.9	2.0	127	14.9	25	121
	1.25	12.1	1.5	145	10.3	19	145

reported increased T/S ratios in hypophysectomized rats fed a Remington (iodine-deficient) diet for 19–20 days (12).

The results of the thyroid iodine analyses presented in Table 2 are in general accord with the theory advanced by VanderLaan and Caplan (13) that there is an inverse relation between the total iodine (I^{127}) concentration and the T/S ratio. This correlation is valid, however, only when hypophysectomized rats are compared with each other, or when control rats are compared with each other. It does not apply when hypophysectomized rats are compared with control rats.

Unblocked thyroids.—In the absence of propylthiouracil, T/S ratios were measured with the aid of the procedure for determining inorganic I^{121}

in the thyroid described under "Methods." Thyroid iodine (I^{127}) analyses were carried out not on the same thyroid glands used for determining T/S ratios, but on thyroid glands of other rats in the same dietary group (Table 3).

It is of interest to compare the *unblocked* T/S ratios shown in Table 3 with the *blocked* T/S ratios shown in Table 2. Because the unblocked T/S ratios appeared to increase with time after the I^{131} injection (Table 3), probably only the results obtained with the shorter intervals can be used

TABLE 3. THYROID I^{127} CONCENTRATIONS AND UNBLOCKED T/S RATIOS IN HYPOPHYSECTOMIZED AND CONTROL RATS

Group	Interval after I^{131}	Hypophysectomized			Control		
		Thyroid weight	T/S	Thyroid I^{127} conc.	Thyroid weight	T/S	Thyroid I^{127} conc.
	hours	mg.		mg. %	mg.		mg. %
A							
Rats placed on low iodine diet after hypophysectomy	3	10.2	2.1		13.6	28	
	3	12.7	1.7		15.0	41	
	96	10.2		125	9.7		87
	96	9.0		160	10.5		79
	96	9.7		162			
B							
Rats placed on low iodine diet 2 weeks before hypophysectomy	28	10.4	4.1		10.2	406	
	28	11.0	5.7		13.2	184	
	0.75	11.3		96	11.2		74
	0.75	8.8		106	14.9		45
	0.75	7.8		98	13.0		65
Rats placed on low iodine diet 5 weeks before hypophysectomy	0.75	12.3	19		12.9	9.0	
	0.75	6.3	10		11.3	14	
	0.75	10.0	11.5		9.1	24	
	29	8.7		74	12.7		52
	29	6.1		111	14.0		71
C							
Rats maintained on Purina-base diet throughout	78	10.4	2.6		17.9	160	
	78	12.0	9.2		21.0	260	
	6	15.2		146	16.1		94
	6	10.4		148	13.8		139
	6				14.1		142

for valid comparison with the blocked T/S ratios. On this basis, the unblocked T/S ratios in the control rats (Group A, 3 hours; Group B, 0.75 hours; Table 3) are much lower than the corresponding blocked T/S ratios. This confirms the findings of Wollman and Scow who, on the basis of such results, concluded that the iodide trap is the rate-limiting step in the overall conversion of iodide to hormonal iodine (8). It is possible that the high T/S ratios observed at the later intervals in unblocked glands may be partly due to iodide within the gland released by deiodination, although Ingbar and Freinkel (14) concluded from their studies that deiodination does not contribute materially to the dialyzable iodine within the thyroid.

Further work is apparently necessary to establish the significance of these high T/S ratios.

DISCUSSION

Although there can be little doubt from previous results obtained in this laboratory (1, 15) and elsewhere (16–22, and others) that thyroid hormone secretion is greatly depressed in the absence of the pituitary, there is little quantitative information available on the magnitude of this depression.

We have attempted to estimate, from the data of the present investigation, the actual rate of formation of thyroglobulin by the thyroids of hypophysectomized and control rats. This calculation is complicated by the following factors: 1) The magnitude of the extrathyroidal iodide pool is probably different in control and hypophysectomized rats; 2) the rate of labeling of the thyroid iodide pool (as well as its size) may differ in control and hypophysectomized rats; and 3) injected I^{131}-iodide disappears from the extrathyroidal iodide pool at a greater rate in control rats than in hypophysectomized rats. We have attempted to overcome some of these difficulties by 1) using rats fed a low iodine diet, 2) administering 5 μg. of iodide carrier along with the I^{131}, and 3) basing the calculations on measurements made at an early interval after the I^{131} injection. The injected 5 μg. of iodide is probably larger than the total iodide pool of rats on the low iodine diet, and we can assume, therefore, that in the interval immediately following the injection the specific activity of circulating iodide is much the same in control and operated rats. Measurements were made, therefore, at an early interval (45 minutes) after the injection, a procedure which also minimized the effect of the greater disappearance rate of I^{131}-iodide from the iodide pool of control animals. With regard to the rate of labeling of the thyroid iodide pool, the values in Table 2 for the T/S ratios in the rats of Group A are of interest. In this group, the blocked T/S ratios were measured at various times after I^{131} injection, and in the hypophysectomized rats there was no change in the T/S ratio from 1.25 to 8 hours, suggesting a fairly rapid equilibration between thyroidal iodide and extrathyroidal iodide. In the control rats, the blocked T/S ratio appeared to increase with time, but this was not confirmed in a subsequent experiment employing a larger number of animals.

The values shown in Table 1 indicate the number of micrograms of *injected* iodide converted to thyroglobulin during the 45-minute interval after the injection. The values for the control rats averaged approximately 0.1 μg., but those for the hypophysectomized rats averaged only about .001 μg. However, the degree to which these values reflect the actual quantity of iodide converted to thyroglobulin depends upon the validity of the considerations discussed in the preceding paragraph. Insofar as the foregoing assumptions are applicable, the present results indicate that thyro-

globulin formation in hypophysectomized rats is reduced to about 1 percent of the control value.

Not only was the rate of thyroglobulin formation greatly diminished in the absence of the pituitary, but also the thyroglobulin which was formed under these conditions appeared to have a greatly reduced thyroxine content. This was evidenced by the finding that labeled thyroglobulin in the thyroids of hypophysectomized rats contained only 5–10% as much I^{131}-thyroxine as did the labeled thyroglobulin in control thyroids. However, it must be pointed out that comparisons of the amount of thyroxine formed in normal and hypophysectomized animals, based on I^{131} data alone, are valid only insofar as there are no large differences between such animals in the distribution of stable iodine (I^{127}) within their thyroids. Specific activities of I^{131} in the various iodine fractions in the gland were not measured in the present work. In a previous study, however, chemical iodine determinations were performed on the thyroids of normal and hypophysectomized rats, and no significant differences were observed in the distribution of stable iodine within the gland (15). The fractionation of thyroid iodine in that study was carried out by a butanol extraction procedure, after the tissue had been hydrolyzed with 2N NaOH. Although these procedures are now known to be inadequate, they nevertheless suggest that there are no *large* differences between the thyroids of normal and hypophysectomized rats in the distribution of chemical iodine. It is not likely, therefore, that the very large depression of I^{131}-thyroxine formation which occurred in the thyroids of rats after hypophysectomy was merely a reflection of a large increase in the pool size of a thyroxine precursor.

The absence of the pituitary had the same inhibitory effect on the formation of I^{131}-triiodothyronine as it had on I^{131}-thyroxine formation. At all intervals after I^{131} injection (up to 8 days), I^{131}-triiodothyronine was barely detectable in the thyroids of hypophysectomized rats. The monoiodotyrosine and diiodotyrosine contents of the newly formed thyroglobulin, on the other hand, did not appear to be reduced by hypophysectomy. In fact, the I^{131}-monoiodotyrosine content was increased, suggesting that hypophysectomy had more effect on the conversion of mono- to diiodotyrosine than on the formation of monoiodotyrosine.

The plasma of hypophysectomized rats contained no significant amounts of I^{131}-thyroxine (or other iodothyronines) even as late as 8 days after I^{131} injection, whereas in control plasma, I^{131}-thyroxine was easily detectable after 6 hours (Figure 7). These observations provide further evidence that thyroxine secretion is greatly depressed in the absence of the pituitary. The failure of I^{131}-thyroxine to appear in the plasma of hypophysectomized rats is not surprising in view of the results described above—i.e., 1) the greatly depressed rate of thyroglobulin formation by the thyroids, and 2) the very low thyroxine content of the thyroglobulin which was formed.

Our findings do not appear to be in quantitative agreement with those

of Randall *et al.* (17). These workers measured the disappearance rate of I^{131} from the thyroids of normal and hypophysectomized rats and, assuming that all of the I^{131} which disappeared from the gland was in the form of thyroxine, they concluded that thyroxine secretion in the absence of the pituitary was reduced to approximately $\frac{1}{5}$ the normal rate. Our findings, on the other hand, suggest a reduction of a hundredfold or more.

Results recently reported by Evans *et al.* (23) support the conclusion that thyroxine formation in hypophysectomized rats is reduced to extremely low levels. These workers found that the BMR of hypophysectomized rats was reduced to a level as low as that reached after complete thyroidectomy (−50%). Moreover, the minimum level was attained more rapidly after hypophysectomy than after thyroidectomy.

There is biological evidence, however, which suggests that the thyroids of hypophysectomized rats are not completely devoid of function. Scow (24), for example, found that, whereas hypophysectomized rats grew at a small but significant rate, rats that were both hypophysectomized and thyroidectomized did not grow at all. Furthermore, growth could be induced in the doubly operated animals by administration of minute amounts of thyroxine. Asling (25) reported that the skeletal response to prolonged injections of growth hormone is much greater in hypophysectomized than in thyroidectomized rats, and that administration of thyroxine to the thyroidectomized rats improved the skeletal response to injected growth hormone. The experiment of Asling, however, is open to the criticism that the growth hormone may have contained a small TSH contaminant or, possibly, that growth hormone itself, over a prolonged period, may have stimulated the thyroid directly.

Although I^{131}-thyroxine formation was greatly depressed in the thyroids of the hypophysectomized rats studied here, we do not wish to imply that this is the primary site of action of TSH on the thyroid gland. A discussion of the mechanism of action of TSH on the thyroid gland appears in the following paper.

The striking decrease in the T/S ratio previously reported to occur in rats following hypophysectomy (8, 10, 11) is confirmed in the present investigation. Moreover, the dependance of the absolute value of the T/S ratio on the diet fed to the rats before removal of the pituitary is also demonstrated. Presumably, the iodine content of the diet is the most important factor involved here, although this was not proved in the present study. If this be assumed, however, there are at least two mechanisms by which previous iodine intake might affect T/S ratios in hypophysectomized rats:

1) The feeding of a low iodine diet to rats with intact pituitaries results in an increased rate of secretion of TSH by the pituitary. This, in turn, produces changes in the thyroid gland, including in the terminology of Halmi (26), an increase in the number of iodide acceptor sites. After hypoph-

ysectomy, with consequent removal of TSH, there is a gradual decrease in the number of iodide acceptor sites. However, an appreciable number of these remain, even after 10 days, primarily because of their initially high level. In rats transferred to the low iodine diet after hypophysectomy, there is no stimulation of the thyroid by excess TSH, and consequently little or no change in the number of iodide acceptor sites.

2) Halmi (27) and VanderLaan (28) have considered the problem, to what extent the regulation of thyroid iodine metabolism is independent of TSH. Both these workers suggest that the thyroid possesses a considerable degree of autonomy, and that the concentration of total iodine (VanderLaan), or of some particular organic iodine component (Halmi), within the gland, exerts an important influence on the iodide pump. On this basis, the higher T/S ratios observed in hypophysectomized rats that had been fed a low iodine diet before hypophysectomy are related to the lower concentration of iodine within the thyroid at the time of hypophysectomy.

Which of the above mechanisms is primarily responsible for the observed results cannot be definitely stated. Perhaps both are involved to some extent. It is of interest that in our experiments the feeding of a low iodine diet *after* hypophysectomy had little or no effect on T/S ratios (Table 2), whereas Halmi (12) previously found that the feeding of an iodine deficient (Remington) diet for 19–20 days to hypophysectomized rats increased T/S ratios appreciably.

The profound depression in all phases of thyroid iodine metabolism which occurred in all of the hypophysectomized rats studied here leads us to minimize the importance of autonomous thyroid regulation and to emphasize the importance of pituitary control. As discussed in more detail in the following paper, we suggest that TSH acts on some basic thyroid cellular process which in turn affects each step in thyroid iodine metabolism. As might be expected, the response of the thyroid to TSH is subject to modification by a variety of factors (29, 30), but our evidence indicates that TSH is by far the most important single factor in the physiological regulation of thyroid function.

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THYROID I^{131} METABOLISM IN THE ABSENCE OF THE PITUITARY: THE HYPOPHYSECTOMIZED RAT TREATED WITH THYROTROPIC HORMONE[1]

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ABSTRACT

Hypophysectomized rats that had been treated with thyrotropic hormone (TSH) for varying periods were injected with 5 μg. of iodide labeled with I^{131}. Treatment with TSH readily reversed the abnormal distribution of I^{131} (low thyroxine I^{131}, high monoiodotyrosine-I^{131}, which occurs in the thyroids of hypophysectomized rats. Thyroids removed from hypophysectomized rats that had received injections of TSH showed greater ability to concentrate iodide *in vitro* than did thyroids removed from saline injected hypophysectomized rats. The mechanism of action of TSH on the thyroid is discussed. Evidence is presented which indicates that the effects of TSH on both the thyroid iodide pump and on thyroid cellular growth are independent of any effect on iodine discharge. It is suggested that the various steps of thyroid iodine metabolism are affected by TSH independently. The effects of TSH on iodine metabolism are probably secondary to its effect on some basic cellular process in the thyroid.

THE preceding paper (1) describes certain defects in thyroid I^{131} metabolism which occur in hypophysectomized rats. The present report deals with the reversal of these defects by the administration of thyrotropic hormone (TSH). Also presented here are the results of several other experiments pertaining to the mechanism of action of TSH on the thyroid gland.

METHODS

Three different types of experiments are reported in the present study: 1) *in vivo* experiments with untreated and with TSH-injected, hypophysectomized rats; 2) *in vitro* experiments with thyroids removed from untreated and from TSH-injected, hypophysectomized rats; and 3) investigation of the effects of hypophysectomy and of hypophysectomy plus TSH on the thyroids of rats previously made goitrous by the chronic feeding of propylthiouracil. Methods are described separately below for each type.

A. *In Vivo Experiments with Hypophysectomized Rats Injected with TSH.*—These rats were similar in all respects to those described in Group A of the preceding paper (1). Injection with TSH[2] was carried out according to three different dosage schedules:

1) In one experiment, hypophysectomized rats received a total of three subcutaneous injections of TSH, spaced at intervals of approximately 12 hours. The injections were

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[2] Parke, Davis and Co. Preparation R_x 099931. It had not been quantitatively assayed.

started nine days after surgery, and consisted of 10 mg. for the first injection, and 5 mg. each for the second and third injections. Hypophysectomized control rats received saline only. One to two hours after the last TSH injection, each rat was injected intraperitoneally with 75 μc. of I^{131} containing 5 μg. of iodide. The animals were sacrificed three hours later, and thyroids from two rats were pooled for chromatographic analysis as described in the preceding paper (1).

2) In a second experiment, rats were injected subcutaneously with TSH twice daily for two days and once on the third day. The injections were started five days after surgery and were administered approximately 12 hours apart, each injection consisting of 5 mg. of TSH in 0.25 ml. of saline. Hypophysectomized control rats received saline only. Thirty to 90 minutes after the last TSH injection, 25 μc. of I^{131} containing 5 μg. of iodide were injected intraperitoneally, and the animals were sacrificed three hours later. Thyroids from two rats were pooled for chromatographic analysis as described in the preceding paper (1).

3) In another series of three experiments, the effect of a single dose of TSH was determined. Eight days after surgery, hypophysectomized rats were injected subcutaneously with 10–20 mg. of TSH in 0.5 ml. of saline. Hypophysectomized control rats received saline only. Four hours later, all rats received I^{131} containing 5 μg. of iodide. Three hours after the I^{131} injection, the thyroids were removed and analyzed chromatographically as described in the preceding paper (1) for thyroids from a single rat.

B. *In Vitro Experiments with Thyroids Removed from Hypophysectomized Rats Treated with TSH.*—The rats used for these studies were similar in all respects to those described in Group A of the preceding paper (1). Beginning seven days after removal of the pituitary, they received three subcutaneous injections of TSH (Parke, Davis Co. Prep. R_x 099931) spaced at intervals of approximately 12 hours. The first injection contained 10 mg., and each of the second and third injections 5 mg. of TSH. Hypophysectomized control rats received saline injections. Three to four hours after the last injection, the thyroids were removed and weighed, and each lobe was sliced longitudinally into approximately equal parts. The four thyroid sections from each rat were then transferred to a 10-ml. Erlenmeyer flask containing 1.5 ml. of oxygenated Krebs-Ringer-bicarbonate buffer including 0.6 μg. of iodide and 20–25 μc. of purified I^{131}. Samples of tissue were incubated, with shaking, for 75 minutes at 37°, some in the presence, and some in the absence of 10^{-3} *M* thiouracil.

At the end of the incubation period, the flask contents were poured into a dry beaker. The small pieces of thyroid tissue were removed with a pair of fine forceps and rinsed briefly in nonradioactive Krebs-Ringer-bicarbonate solution. For samples incubated in the presence of thiouracil, the tissue was dissolved in alkali, and aliquots of both tissue and medium were assayed directly for radioactivity in the well-type scintillation counter.

The samples in which organic binding had not been blocked were homogenized in a dry, conical, motor-driven, glass homogenizer, at 0°, with 400 μl. of Krebs-Ringer-bicarbonate buffer containing 10 μl. of 0.001 *M* thiouracil. 100 μl. of the homogenate were treated with 200 μl. of ethanol for the determination of tissue iodide-I^{131}, as described in the preceding paper (1). 20 μl. of the homogenate were chromatographed directly, and the remainder was digested with pancreatin for determination of the individual iodinated amino acids (1).

C. *Experiments with Rats Made Goitrous by the Feeding of Propylthiouracil.*—These experiments were performed to determine whether TSH would affect the thyroid weight and T/S ratio of thyroids previously depleted of almost all their iodine by the feeding of a diet containing PTU, and deprived of endogenous TSH stimulation by hypophysectomy. Five separate experiments were carried out with Long-Evans rats weighing 150–220 gm. Female rats were used in four experiments, male rats in one. In each experiment the animals were fed, for 15–19 days, the low iodine diet described in the preceding

paper, modified to include 0.1% PTU. At the end of that time, some of the rats were sacrificed while all others were hypophysectomized. The latter were continued on the PTU diet. T/S ratios, thyroid weight, and, in most cases, thyroid iodine (I^{127}) content were determined at 0 time and at intervals after hypophysectomy.

Many of the rats received TSH injections starting nine to eleven days after removal of the pituitary. Each rat received seven injections of TSH, 3–4 mg. each, administered subcutaneously approximately every 12 hours. T/S ratios and thyroid weight were determined two to four hours after the last TSH injection.

Assay of TSH.—Three different TSH preparations were used. They were bioassayed for the presence of other pituitary hormones with the following results: 1) Parke, Davis TSH Lot 50P4: positive for FSH at 0.5 mg. total dose (follicle growth test of Evans *et al.* (2)); positive for ICSH at 0.25 mg. total dose (interstitial cells repair method of Simpson *et al.* (3)); negative for ACTH (adrenal repair test of Simpson *et al.* (4)); negative for growth hormone at 3 mg. total dose (rat tibia test of Greenspan *et al.* (5)). 2) Armour TSH Lot P589–80: positive for FSH at 3 mg. total dose; positive for ICSH at 0.5 mg. total dose; negative for ACTH at 3 mg. total dose; negative for growth hormone at 3 mg. total dose. 3) TSH prepared by the method of Fels *et al.* (6, 7): negative for FSH at 3 mg. total dose; positive for ICSH at 5 mg. total dose; negative for ACTH at 3 mg. total dose; negative for growth hormone at 3 mg. total dose.

All the above TSH preparations had a minimum effective dose of approximately 10μg., based on the minimum amount of material which increased thyroid I^{131} uptake and produced changes in thyroid cytology in immature, female, hypophysectomized rats (6).

For the determination of T/S ratios, each rat was injected with 16 mg. of PTU to ensure complete inhibition of organic binding (even though all rats had been fed the PTU-containing diet). Thirty minutes later, each rat received an injection containing 1 μc. of I^{131} and 0.5 μg. of stable iodide. Thyroids were removed 1 hour after the I^{131} injection, and a sample of blood was obtained simultaneously by cardiac puncture. Thyroids were first assayed for I^{131} *in toto* in the well-type scintillation counter, and then they were transferred to digestion flasks for determination of total iodine (I^{127}) by a method previously described (8).

RESULTS

I. *Effect of Multiple Injections of TSH on Distribution of I^{131} in Thyroids of Hypophysectomized Rats*

The results in Table 1 confirm the findings presented in the preceding paper that the distribution of I^{131} in the thyroids of hypophysectomized rats is markedly different from normal. Injection of 20 mg. of TSH in three doses over a period of 24 hours restored the distribution almost to normal (Experiment 1). This was particularly evident in the fraction of total I^{131} present as thyroxine; this value was elevated from an average of 0.5% in the saline-treated, hypophysectomized rats to an average of 5.0% after treatment with TSH. It may be noted also that the ratio

$$\frac{\text{diiodotyrosine } I^{131}}{\text{monoiodotyrosine } I^{131}}$$

was also augmented by treatment with TSH. The fraction of the total I^{131} present as inorganic iodide was reduced by TSH injection.

TABLE 1. EFFECT OF THYROTROPIC HORMONE INJECTIONS ON DISTRIBUTION OF I^{131} IN THYROIDS OF HYPOPHYSECTOMIZED RATS

(All rats sacrificed 3 hours after I^{131} injection. See text for details)

	Experiment 1			Experiment 2		
	Saline-injected HypX	TSH injected HypX*	Untreated control	Saline-injected HypX	TSH-injected HypX†	Untreated control
1. No. of animals in group	2	6	2	4	4	4
2. Average thyroid weight (mg.)	11.5	12.9	16.0	11.4	20.2	14.3
3. Average uptake of I^{131} (per cent of injected dose)	0.10	2.4	13.8	0.15	14.7	11.9
4. Distribution of I^{131} in thyroids before hydrolysis. Average per cent of thyroid I^{131} in:						
Thyroglobulin	83.4	92.0	96.8	87.6	93.6	96.4
Inorganic iodide	16.6	7.8	3.0	12.3	6.4	3.6
5. Distribution of I^{131} in thyroids after enzymatic digestion. Average per cent of thyroid I^{131} in:						
Thyroxine	0.5	5.0	9.5	0.5	6.9	7.8
Diiodotyrosine	34.0	42.2	42.7	42.4	41.6	45.3
Monoiodotyrosine	41.6	35.1	31.4	36.3	26.1	26.7
Inorganic iodide	19.1	8.2	5.5	14.6	11.7	7.4
Triiodothyronine		0.9	1.4		2.3	1.6
Undigested thyroglobulin	2.9	4.6	5.3	3.1	6.1	5.3
Unidentified	1.9	3.9	4.2	3.0	5.2	5.6

* Three injections of TSH. See text for details.
† Five injections of TSH. See text for details.

Administration of 25 mg. of TSH in five doses over a period of 48 hours (Experiment 2, Table 1) completely restored to normal not only the distribution of I^{131} in the thyroids of hypophysectomized rats but also the total thyroid uptake of I^{131}. Thyroid weights were increased beyond the normal range by treatment with this dosage of TSH.

II. *Effect of a Single Injection of TSH on Distribution of I^{131} in Thyroids of Hypophysectomized Rats*

As shown in Table 2, a single injection of TSH appeared to have a significant effect on the percentage of the total I^{131} present as thyroxine in the thyroids of hypophysectomized rats. This effect was apparent seven hours after the TSH injection. No experiments were performed to determine whether an effect occurred at earlier intervals.

A single injection of TSH also had a noticeable effect on the ratio

$$\frac{\text{diiodotyrosine } I^{131}}{\text{monoiodotyrosine } I^{131}}.$$

In each experiment this ratio was higher in the TSH-treated than in the saline-treated, hypophysectomized rats. Evidently, increased conversion of mono- to diiodotyrosine and of diiodotyrosine to thyroxine occurred within seven hours after a single large injection of TSH. Thyroid weight, on the other hand, was unaffected in this interval, and the increase in total I^{131} uptake was not significant ($P < 0.05$).

TABLE 2. EFFECT OF A SINGLE INJECTION OF TSH ON THE DISTRIBUTION OF I^{131} IN THE THYROIDS OF HYPOPHYSECTOMIZED RATS

	Saline-treated	TSH-treated
No. of rats	5	8
Thyroid weight (mg.)	10.8* ±1.0	10.5* ±0.78
Uptake of I^{131} (per cent of injected dose)	0.066 ±0.012	0.13 ±0.024
Distribution of I^{131} in thyroids before hydrolysis. Per cent of thyroid I^{131} in:		
Thyroglobulin	84.2 ±2.7	91.5 ±0.75
Inorganic iodide	15.8 ±2.7	8.5 ±0.75
Distribution of I^{131} in thyroids after enzymatic digestion. Per cent of thyroid I^{131} in:		
Thyroxine	0.86 ±0.10	2.2 ±0.16
Diiodotyrosine	34.6 ±1.7	44.5 ±0.75
Monoiodotyrosine	40.0 ±1.8	33.9 ±0.82
Inorganic iodide	16.5 ±3.0	9.7 ±0.50
Undigested thyroglobulin	4.9 ±0.80	5.5 ±0.50

* Mean ±standard error.

III. *In Vitro Experiments with Thyroids from Hypophysectomized Rats*

As shown in Table 3, thyroids removed from hypophysectomized rats that had received injections of TSH showed greater ability to concentrate iodide *in vitro* than did thyroids removed from control hypophysectomized rats. This was true whether or not organic binding was blocked by thiouracil. Based on data from two separate experiments, the T/M ratio in the presence of thiouracil was 2.2 ±0.21 (mean+S.E.) for thyroids from control hypophysectomized rats, and 14.4 ±2.1 for thyroids from hypophysectomized rats that had received three injections of TSH. In the absence of thiouracil, the corresponding ratios were 2.2 ±0.18, and 7.4 ±0.90, respectively. The total uptake of I^{131} was also considerably higher in the thyroids from TSH-treated rats.

Similar experiments with thyroids from hypophysectomized rats that had received a single injection of TSH did not show any consistent effects on T/M ratios or total I^{131} uptake.

The distribution of I^{131} in rat thyroids incubated *in vitro* was quite different from that observed *in vivo*. Relatively more of the I^{131} taken up by

TABLE 3. *In vitro* UTILIZATION OF RADIOIODIDE BY THYROIDS REMOVED FROM SALINE-INJECTED AND FROM TSH-INJECTED HYPOPHYSECTOMIZED RATS

(See text for details, page 665)

Treatment	10^{-3} M thiouracil in medium		No thiouracil in medium				
	Uptake of I^{131} by tissue	T/M*	Uptake of I^{131} by tissue	T/M*	Per cent of tissue I^{131} in: Monoiodotyrosine fraction	Diiodotyrosine fraction	Iodide fraction
	per cent of added I^{131} per 100 mg. of tissue		per cent of added I^{131} per 100 mg. of tissue				
Saline-HypX (6)	14.2±1.3†	2.2±0.21	29.7±4.9	2.2±0.18	18.5±1.2	9.4±1.3	57.5±3.0
TSH-HypX (6)	80.5±9.8	14.4±2.1	62.3±6.9	7.4±0.90	13.8±0.90	5.0±0.65	74.7±2.7
Normal (3)	152±25	30.3±6.3	145±27	15.3±4.2	23.2±1.4	11.1±1.8	56.8±4.0

Figures in parentheses refer to number of animals in group.

* $\frac{\text{Iodide-}I^{131}\text{ concentration in tissue}}{\text{Iodide-}I^{131}\text{ concentration in medium}}$

† Mean ± standard error.

the tissue in the former case remained as inorganic iodide, and much less was converted to iodotyrosines (Table 3). I^{131}-thyroxine was formed *in vitro* only in traces, even in thyroids removed from TSH-treated rats. The effect of previous treatment with TSH appeared to be an increase in the fraction of the thyroid I^{131} remaining as inorganic iodide. It is of interest that under the *in vitro* conditions employed here, the distribution of I^{131} in thyroids from hypophysectomized rats was not significantly different from that observed in thyroids from normal rats. This may be contrasted with the *in vivo* results reported in the preceding paper (1). Only the iodide trap and its response to TSH yielded results *in vitro* which paralleled those observed *in vivo*. Evidently the enzymes or other factors concerned with organic iodine formation are more sensitive to a change from *in vivo* to *in vitro* conditions than are those factors concerned with concentrating iodide.

IV. *Effects of Hypophysectomy and of Hypophysectomy plus TSH on the Thyroids of Rats Previously Made Goitrous by the Chronic Feeding of Thiouracil*

The results of four separate experiments are shown in Figure 1 and in Table 4. The findings may be summarized as follows: The feeding of 0.1% PTU for 15–19 days raised the average T/S ratio to a value of more than 300 and lowered the concentration of I^{127} in the gland to 1–2 mg. per cent. (The same diet without PTU, fed to rats for 15 days, resulted in T/S ratios of 60–80 and thyroid I^{127} concentrations of 50–60 mg.%) Removal of the pituitary significantly lowered the T/S ratio within four days (Figure 1) and by eight days, the T/S ratios averaged less than one fourth of the initial value. The thyroid I^{127} concentration, on the other hand, did not change significantly. Administration of TSH to the hypophysectomized rats restored the T/S ratio almost to the initial value, while it had little effect on the thyroid I^{127} concentration. It is clear from these experiments that TSH had a pronounced effect on the iodide-concentrating capacity of the thyroid, while at the same time it had little, if any, effect on the iodine concentration within the gland. The bearing of these results on the mechanism of action of TSH on the thyroid gland will be discussed below.

DISCUSSION

The TSH used for most of the experiments reported here contained appreciable amounts of other pituitary hormones. Since relatively large doses of TSH were administered, the question arises to what extent our results were influenced by other endocrine factors. As far as we know, stimulation of the thyroid by short-term treatment with ACTH or with growth hormone has not been reported, but short-term treatment with estrogen was reported by Feldman (9) to increase T/S ratios in hypophysectomized rats. It is unlikely, however, that estrogen formation played any role in our results since each type of experiment was carried out either

TABLE 4. EFFECT OF HYPOPHYSECTOMY AND OF HYPOPHYSECTOMY PLUS TSH ON THE T/S RATIO AND THYROID IODINE CONCENTRATION OF RATS PREVIOUSLY MADE GOITROUS BY THE FEEDING OF A DIET CONTAINING 0.1 PER CENT PROPYLTHIOURACIL

	Days after hypophysectomy											
	0			5			14 Saline injections last 3 days			14 TSH injections last 3 days		
	T/S	Thyroid I^{127}	Thyroid weight	T/S	Thyroid I^{127}	Thyroid weight	T/S	Thyroid I^{127}	Thyroid weight	T/S	Thyroid I^{127}	Thyroid weight
		mg. per cent	mg.		mg. per cent	mg.		mg. per cent	mg.		mg. per cent	mg.
Exp. 2	343 ± 57 (9)	0.92 ± 0.07 (4)	44.8 ± 2.6 (9)				62 ± 23 (2)	2.0 ± 0.21 (2)	14.5 ± 1.0 (2)	339 ± 98* (4)	1.5 ± .33 (4)	28 ± 2.0 (4)
Exp. 3	319 ± 33 (7)	1.0 ± 0.14 (7)	64.4 ± 4.5 (7)	71 ± 7.5 (4)	1.8 ± 0.32 (4)	26 ± 3.9 (4)	47 ± 2.9 (3)	3.0 ± 0.11 (3)	15.7 ± 1.2 (3)	197 ± 41† (5)	1.0 ± 0.10 (5)	38.7 ± 4.2 (5)
										238 ± 18†‡ (4)	1.4 ± 0.29 (7)	33.3 ± 3.0 (4)
Exp. 4	200 ± 28 (5)		58.3 ± 3.3 (5)				58 ± 0.92 (4)		14.5 ± 0.92 (4)	236 ± 42* (5)		38.3 ± 5.5 (5)

Figures in parentheses indicate number of rats per group.
* Parke, Davis and Co. TSH, Prep. R_x 50P4. See assay under "Methods."
† Armour and Co. TSH Prep. P589-80. See assay under "Methods."
‡ TSH prepared by method of Fels (6, 7). See assay under "Methods."

exclusively, or at least in part, with male rats. Furthermore, as shown in Table 4, results obtained in one experiment (Experiment 3) with a highly purified TSH preparation were comparable to those obtained with more contaminated TSH preparations. While no final answer can be given to the part played in our experiments by endocrine factors other than TSH, it appears likely that at least the major part of the effects observed here was due to TSH itself. In the discussion which follows we assume that this is the case.

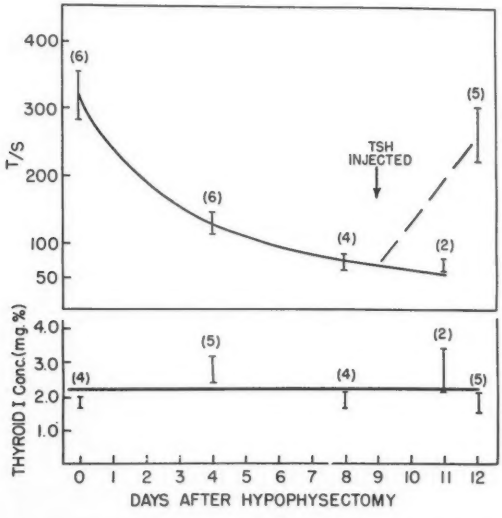

FIG. 1. T/S ratio (upper curve) and total thyroid iodine concentration (lower curve) in rats at various intervals after hypophysectomy. All rats had been fed a diet containing 0.1 per cent PTU for 15 days before removal of the pituitary. TSH was administered twice daily for 3 days starting 9 days after hypophysectomy. Figures in parentheses indicate number of rats in each group. Vertical lines indicate standard errors.

The changes which occur in thyroid I^{131} metabolism in rats after complete hypophysectomy are summarized in the preceding paper. The present communication describes the reversal of these changes by the administration of TSH. Particularly emphasized here is the ability of TSH to restore to normal the distribution of I^{131} in the thyroids of hypophysectomized rats. The greatly depressed fraction of total thyroid I^{121} present as thyroxine in hypophysectomized rats was noticeably increased within seven hours after a single large injection of TSH. Three injections of TSH restored the I^{131}-thyroxine content of newly formed thyroglobulin almost to normal. Absence of the pituitary also lowered the ratio

$$\frac{\text{diiodotyrosine-}I^{131}}{\text{monoiodotyrosine-}I^{131}}$$

in the thyroids of rats, suggesting that the conversion of monoiodotyrosine to diiodotyrosine is impaired. This ratio was restored to the normal range within seven hours after a single large injection of TSH.

The effect of TSH on the thyroid iodide trap was studied by VanderLaan & Greer (10), by Halmi and his coworkers (11), and by Wollman & Scow (12). Their results, together with those described in this and in the preceding paper, indicate that, in the rat, the thyroid iodide trap is reversibly affected by TSH. Halmi *et al.* (11) reported that T/S ratios in hypophysectomized rats are significantly increased five to six hours after a single injection of TSH.

It is of interest that thyroids removed from hypophysectomized rats that have been treated with TSH still retain *in vitro* an increased ability to take up iodine and to concentrate iodide (Table 3). This indicates that TSH produces some change in the thyroid which persists after the tissue is removed from the animal. It does not seem likely, therefore, that the effect of TSH on the iodide trap is secondary to the increased blood flow rate through the thyroid which accompanies TSH action. The increased blood flow rate to the thyroid, commonly observed after treatment with TSH, is probably secondary to the increased metabolism of the tissue.

In vitro studies with thyroids removed from TSH-treated rats were previously made by Botkin and Eskelson and their coworkers (13, 14). They injected TSH into normal (14) as well as into hypophysectomized (13) rats, and studied the I^{131} metabolism of the isolated thyroids. Their results with hypophysectomized rats were similar to those reported here, but with normal rats, they found that TSH injection depressed the I^{131} uptake of the isolated thyroids. The significance of the latter observation remains to be determined.

Many workers have demonstrated an effect of TSH on the release of thyroid hormone from the thyroid gland (15–23; see (23) for clinical references), and this has been suggested as the primary site of action of TSH (17, 24, 25). According to this view, an increase in the level of circulating TSH induces a relative iodine deficiency in the thyroid, and this in turn, presumably by displacement of the chemical equilibrium, accelerates all other phases of iodine metabolism within the gland. The evidence for this theory is that the earliest effect of TSH experimentally detectable by most workers is an increase in the disappearance rate of I^{131} from a previously labeled thyroid gland (15–17, 20–21, and others). These considerations led Halmi and his coworkers (11, 26) to discuss the question whether the effect of TSH on the thyroid iodide trap is secondary to its effect on thyroid hormone discharge. They cited results obtained by VanderLaan and Greer (10) to show that the effect of TSH on the iodide trap is an independent one. The latter workers had found that if rats were first subjected to a PTU-containing diet for two weeks to produce hyperplasia and loss of

iodine stores from the thyroid, and then were hypophysectomized (the goitrogen diet being continued), the T/S ratio fell rapidly, even while the gland remained hypertrophic and low in iodine. We have confirmed these findings in the present study, and have shown, in addition, that administration of TSH effectively restores the T/S ratio in such hypophysectomized rats (Figure 1; Table 4). The thyroid iodine concentration in these animals was only about 2 mg.%, and this was not appreciably affected by TSH treatment. It hardly seems possible, therefore, that the TSH promoted any significant discharge of iodine from the thyroids under these conditions. We are led to conclude, with Halmi, that the action of TSH on the thyroid iodide trap is independent of its effect on thyroid hormone discharge.

In addition to its effect on the T/S ratio, TSH administration also markedly increased thyroid weights in hypophysectomized rats whose thyroids

TABLE 5. EFFECT OF TSH ON THYROIDS OF HYPOPHYSECTOMIZED RATS PREVIOUSLY FED 0.1 PER CENT PTU

Group	Treatment (see p. 673 of text)	Experiment 4			Experiment 5		
		No. of animals	Thyroid weight	T/S	No. of animals	Thyroid weight	Uptake of P^{32} by thyroids
			mg.			mg	per cent I.D. per 100 mg. tissue
1	Saline injections	4	14.5±0.92	58±9.5	5	13.5±1.1	0.10± .016
2	TSH injections	5	38.3±5.5	236±42	4	22.6±2.3	0.34± .004
3	TSH and $NaClO_4$ injections	5	32.2±2.5	0.74±0.05	6	32.7±3.3	0.32± .013

had been depleted of iodine by PTU feeding and who were being maintained on PTU to block any new organic iodine formation (Table 4). Such thyroids, however, still possessed an active iodide trap, and additional experiments were performed, therefore, in which this phase of iodine metabolism, too, was blocked. Rats were maintained on 0.1% PTU for 16 to 19 days, hypophysectomized, and continued on the PTU diet as described above. Ten days after hypophysectomy, the rats were divided into three groups. One group received saline injections, one group TSH injections, and the third group, TSH injections+$NaClO_4$. The $NaClO_4$ was injected three times daily (20 mg. at 9 A.M., 3 P.M., and 9 P.M.), and was also administered in the drinking water as 0.5 per cent solution. The TSH injections (3–4 mg. each) were given twice daily (9 A.M.; 9 P.M.) for three days, and once on the fourth day. As shown in Table 5, TSH induced a marked increase in weight even in those thyroids whose iodine metabolism was completely blocked. In the rats of Group 3, the iodide trap was blocked by $NaClO_4$, organic binding was blocked by PTU, and any effect of TSH on iodine discharge was precluded by previous depletion of the gland's iodine. Yet the thyroids of such rats responded readily with increased growth after TSH administration. *The growth response of the thy-*

roid to TSH, therefore, is not dependent on any effect of TSH on thyroid iodine metabolism.

It may also be noted (Table 5) that P^{32} uptake by the thyroids was increased after TSH injection despite the complete block in thyroid iodine metabolism. This suggests that changes in phosphorus metabolism may be more closely related to the primary action of TSH on the thyroid than are changes in iodine metabolism.

Although there can be little doubt that TSH promotes release of hormonal iodine from the thyroid, its action on the iodide trap and on thyroid weight, as indicated above, is not dependent on iodine release. Evidence for an independent action of TSH on the different steps in thyroid iodine metabolism is further provided by the results described in the preceding paper showing a differential effect of hypophysectomy on the various phases of thyroid iodine metabolism. After hypophysectomy, thyroxine formation was much more depressed than diiodotyrosine formation, and diiodotyrosine formation was more affected than monoiodotyrosine formation. Furthermore, in hypophysectomized rats, the very low formation of thyroxine was associated in some instances with a very weak iodide trap ($T/S = 2-3$), and in others with a relatively strong iodide trap ($T/S > 20$). Results obtained by Wollman and Scow (27) indicate that, in the mouse, hypophysectomy does not depress the thyroid iodide trap to the same extent that it does in the rat, although organic iodine formation is depressed to similarly low levels. These widespread changes in iodine metabolism in response to alterations in the level of TSH are not readily explained by a primary effect of TSH on any single phase of iodine metabolism (see also Stanley and Astwood (28)). In addition to the effects already described, TSH is known to affect such fundamental properties of thyroid tissue as O_2 consumption (29) and synthesis of cytoplasmic RNA (30). We suggest, therefore, that the primary action of TSH on the thyroid is on some basic property of the thyroid cell, and that all its effects on iodine metabolism are secondary.

The fact that TSH exerts such a remarkable effect on the mass of thyroid tissue indicates that it stimulates protein synthesis, and very likely, therefore, enzyme synthesis. TSH could thus influence the formation of the special enzymes required for the various steps of thyroid iodine metabolism, and, in this manner, could affect the various phases of iodine metabolism *independently*. The step most sensitive to changes in the TSH level might depend on species characteristics and on local conditions within the gland, since the response of thyroid tissue to TSH is subject to modification by a variety of factors (PTU, low iodine, previous irradiation of the gland, possibly other endocrine factors, etc. (31, 32)). However, in our estimation, this does not militate against the view that TSH is by far the most important physiological regulator of thyroid function.

Greer (33) has suggested that there are two separate thyrotropins from the pituitary, one concerned primarily with growth of the tissue, the other concerned primarily with iodine metabolism. This proposal, however, has not gained wide acceptance (11, 34). We, too, prefer the more conservative view that only one TSH is elaborated by the anterior pituitary and that failure to obtain equal effects of TSH on the various aspects of thyroid iodine metabolism and on thyroid growth may be attributed to unequal sensitivities of these responses to the action of the pituitary hormone.

Acknowledgment

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NOTES AND COMMENTS

THE EFFECT OF NEPHRECTOMY ON THE DISAPPEARANCE OF EXOGENOUS THYROTROPIN FROM RAT PLASMA

ABSTRACT

Three U.S.P. units of thyrotropin were administered intravenously to nephrectomized rats or sham-operated controls. Blood was collected 15, 60, and 120 minutes later and the plasma bioassayed for thyrotropin. The disappearance of thyrotropin from the blood was significantly slower at all three time intervals in the nephrectomized as compared with the control animals, implicating the kidney as a site of inactivation of the hormone. Kidney slices did not inactivate thyrotropin *in vitro*.

In a previous report (1), data was presented demonstrating that 20–30% of an intravenously-administered dose of thyrotropin was recovered from extracts of saline-perfused rat carcasses 60 minutes after injection of the hormone. At the same time, only 1–2% was recovered in the plasma, 3.5% in the kidney, and 1.4% in the urine. Combined recoveries of thyrotropin from carcass, liver, kidney, blood, and urine added up to about 27% of the administered dose. This compared with a value of 32% recovered in 60 minutes from an extract of the whole rat (minus skin and pituitary gland). Thus, over 60% of the exogenous thyrotropin was apparently inactivated. Thyroidectomy had little effect on recovery of thyrotropin from either plasma or carcass, ruling out the thyroid gland as a major site of inactivation. Since thyrotropin was fixed in the kidney and excreted in the urine, studies were carried out on the possible renal inactivation of this substance.

MATERIALS AND METHODS

Male Sprague-Dawley rats (250–300 gm.) were nephrectomized under ether anesthesia. Immediately after the operation, and while the rats were still anesthetized, 3 U.S.P. units of thyrotropin (U.S.P. Thyrotropin Reference Standard) were injected via the saphenous vein. The animals were exsanguinated 15, 60, and 120 minutes later. Blood was collected from the abdominal aorta in 10 cc. syringes containing 0.2 cc. of 10% sodium citrate. Plasma was prepared and frozen until the bioassay was performed. Control rats were sham-operated and subjected to the identical procedure. The bioassay for thyrotropin was carried out by a method involving thyroidal uptake of I^{131} in thyroid-prefed rats following a single intravenous injection of the diluted plasma (2). The concentration of thyrotropin in U.S.P. units per cc. of undiluted plasma was multiplied by a factor of 2.4% of the body weight (3) to obtain the total content of thyrotropin in the plasma. One experiment was performed in which duplicate 100 mg. samples of fresh and boiled rat kidney tissue slices were incubated for 3 hours in 0.5 cc. of rat plasma containing 0.2 U.S.P. units of thyrotropin. The plasma medium was later bioassayed to determine if inactivation had occurred.

RESULTS AND DISCUSSION

As seen in Table 1, removal of the kidneys resulted in significantly higher recoveries of thyrotropin at all three time periods. The straight line obtained by plotting the logarithm of the per cent recovery against the logarithm of the time after injection of thyrotropin (Fig. 1) is in agreement with the data presented by D'Angelo for normal rats (4). Although use of the logarithmic time coordinate might have no justification beyond the observation that it yields a straight line, it is nevertheless evident from Figure 1 that the per cent recovery of exogenous thyrotropin from plasma does not fall exponentially as a function of time, but declines at an ever-slowing rate. Consequently, the half-life of the

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TABLE 1. RECOVERY OF EXOGENOUS THYROTROPIN FROM THE PLASMA OF NORMAL AND NEPHRECTOMIZED RATS

	Minutes after thyrotropin injection	No. rats	% of administered dose of thyrotropin recovered in plasma (mean ± S.E. of mean)	*t*	P
Sham-operated control	15	4	32.1 ± 2.9	3.74	<0.01
Nephrectomized	15	4	54.4 ± 5.2		
Sham-operated control	60	4	2.02 ± 0.47	5.34	<0.01
Nephrectomized	60	4	16.80 ± 2.73		
Sham-operated control	120	4	0.50 ± 0.07	15.30	<0.001
Nephrectomized	120	4	11.40 ± 0.71		

hormone in blood must be constantly increasing. By interpolating in the curves in Figure 1 at the times for 50% and 25% recovery of thyrotropin, the half-life is estimated as 5 minutes in normal and 24 minutes in nephrectomized rats, increasing to several times these values as points selected for calculation in these curves are shifted to the right. On the basis of these observations, it may be postulated that the disappearance of exogenous thyrotropin from plasma is the resultant of (1) a rapid phase of initial movement of thyrotropin from plasma to extracellular fluid, approaching equilibrium, and (2) further movement of thyrotropin from plasma, depending on the rate of removal of this substance from extracellular fluid by the inactivating tissues. Since the half-life of thyrotropin in plasma increases as the absolute concentration in plasma decreases, it is reasonable to suggest that endogenous thyrotropin may persist in the blood stream far longer than might be suspected from studies on the disappearance of large doses of exogenous thyrotropin.

The difference between the mean recoveries of thyrotropin in nephrectomized and control animals amounted to 22.3% of the administered dose 15 minutes after injection (Table 1). This difference declined to 14.8% in 60 minutes and 10.9% in 120 minutes. In an earlier study (1) it was reported that about 5% of the administered dose was fixed in the kidney in 5 minutes and 3.5% in 60 minutes, with 1.4% appearing in the urine in 60 minutes and 5.3% in 120 minutes. Thus, it would appear that the kidney handles thyrotropin both by excretion and inactivation. In the present study, 67.9% of the administered dose was removed from plasma in 15 minutes in the control rats. The kidneys were responsible for the removal of 22.3% of the administered dose, or 32.8% of the total amount of

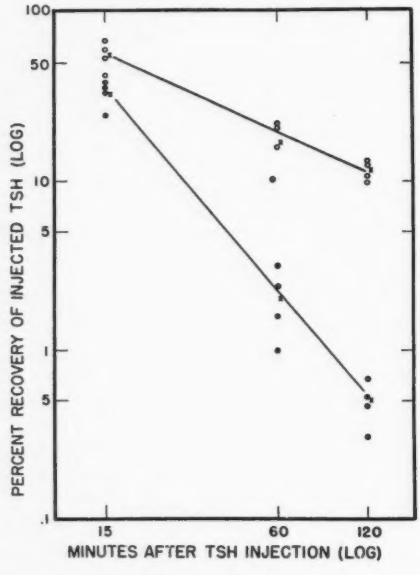

FIG. 1. Recovery of thyrotropin from the plasma of nephrectomized rats (open circles) and sham-operated control rats (solid circles). The average value for the 4 animals in each group is indicated by the cross.

thyrotropin removed from the plasma. At the later times, the differences in thyrotropin recovery between control and nephrectomized rats narrowed somewhat, possibly because other tissues were able to inactivate the hormone which tended to remain in higher concentration in the plasma of the nephrectomized animals.

In the incubation experiment all of the thyrotropin in the medium was recovered after 3 hours, even though the duplicate live kidney slices maintained active respiration for the entire period. It had been previously reported (5, 6) that kidney tissue was incapable of inactivating thyrotropin *in vitro*.

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MORPHOLOGIC CHANGES IN BLOOD VESSELS OF DIABETIC HAMSTERS

ABSTRACT

Saccular distentions were observed in the walls of veins in the cheek pouches of hamsters at 90–120 days after induction of diabetes by treatment with alloxan. The number of vascular irregularities increased with the duration of the disease and were found to be irreversible. Similar morphological changes were observed on arterial vessels after longer periods of diabetes. Evidence is presented which may indicate that these abnormalities are an acceleration of an ageing phenomenon.

Angioscopic changes in the smaller blood vessels in the bulbar conjunctiva have been described in human subjects with diabetes mellitus (1, 2, 3). The purpose of the present investigation was to determine if similar changes occur in a vascular site suitable for study in animals with experimentally-induced diabetes.

METHODS

Twenty-six male and an equal number of female hamsters weighing 90–120 gms. and less than six months of age were fasted overnight and made diabetic with a single intracardiac injection of alloxan (Eastman, a 10% distilled water solution) administered under ether anesthesia. The diabetogenic dose for females was 70–80 mg./kg. and 60–70 mg./kg. for males, in agreement with those reported earlier (4). All animals were force-fed 2 cc. of a 50%

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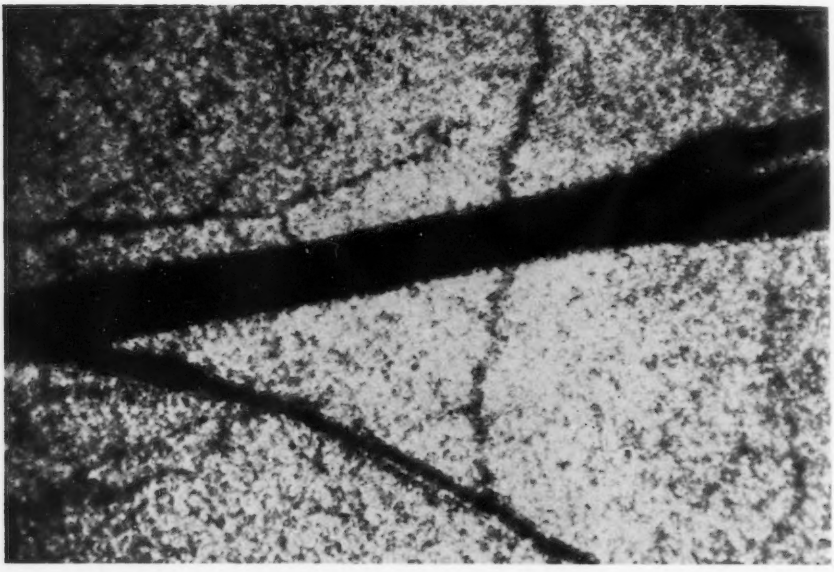

FIG. 1. Vascular sacculations at venous junctions after 120 days of diabetes, ×39.

sucrose solution 6 hours later to prevent hypoglycemic shock. Previous experiments had shown that 40 mg./kg. of alloxan would alter tolerance tests, but were without effect on fasting blood sugar levels (5). Seven animals received this amount of alloxan twice in injections one week apart and served as alloxan-treated controls. Six animals were retained as untreated controls.

Fresh urine samples obtained directly from the bladder were tested daily for the presence of ketone bodies and sugar (Lilly Tes-Tape). Blood sugar concentrations were determined (6) one week after the alloxan injections and once each month thereafter. No attempt was made to regulate blood sugar levels. However, hamsters which had ketone bodies present in urine were treated with 2½ to 3 units of insulin (Lente, Lilly; intramuscularly). Insulin treatment served merely to sustain the animals and prevent fatal acidosis.

Both cheek pouches were examined approximately once a month for a period of one year. Measurements of diameters were made with a screw-micrometer (Leitz) on a major artery and vein in each pouch to obtain A/V ratios. When vascular modifications were observed, diagrams of the vascular pattern were made and the changes noted. Successive observations and a cumulative record of changes revealed that these changes persisted.

RESULTS

Fusiform distentions of the walls of veins resulting in "sacculations" appeared in hamsters rendered diabetic for 90–120 days (Figs. 1 and 2). The sacculations appeared at junctions of veins with larger vessels draining the cheek pouch. They were never observed on collecting venules, capillaries, terminal arterioles or distributing arteries. At least two sacculations were observed in every cheek pouch, and 46% of the animals had more than two (range of 2–6) in each pouch. No vascular abnormalities were observed in the control group.

Distentions appeared in the walls of arteries in the cheek pouches of five of 23 surviving animals after 180 days of diabetes, located at random but limited to major arteries. The number of venous sacculations had increased at this time. Three or more (range of 3–9) were found in each cheek pouch in 65% of the hamsters. Morphological changes in veins also appeared in the controls at this time. Two of the six untreated animals had

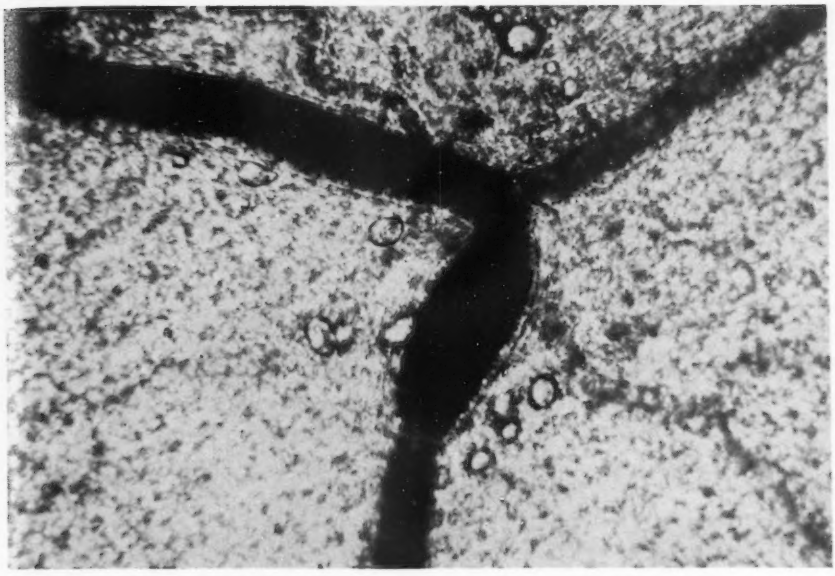

FIG. 2. Sacculation at venous junction, ×77.

developed a single sacculation in each pouch. None of the alloxan-treated controls showed vascular abnormalities.

Eight of the ten diabetic hamsters which survived for 120 days reverted to normoglycemia at that time. Examination of the cheek pouches of these animals four months later showed that the number and location of the sacculations had not changed. Several additional venous distentions had developed in three animals.

One hamster remained diabetic for 370 days. Ten venous and one arterial sacculation were found in one cheek pouch and eleven venous sacculations were found in the opposite pouch upon examination on the 365th day of diabetes. The number of sacculations and the number of animals developing sacculations increased in the control groups at this time. Thus, four (three non-treated) out of ten control hamsters now had a single sacculation in each pouch. Two animals had two venular sacculations in each pouch, and a third had five in one of its cheek pouches.

The ratio of arteriolar to venular diameters (A/V) remained between 1:2 and 1:3 in all groups during the entire observation period.

DISCUSSION

Sacculations of the walls of veins which appeared in the cheek pouches of hamsters made diabetic with alloxan resemble the morphologic changes in veins described by Ditzel (1, 2, 3) in the bulbar conjuctiva of diabetic human subjects. However, other vascular and hemodynamic changes found in man were not observed in the hamster. The causative factors and mechanisms which led to the morphological manifestations are unknown. Although statistical evidence is lacking, morphological changes in veins appear to be a characteristic ageing phenomenon, at least in the hamster, accelerated by diabetes. These findings are consistent with those reported in man (1).

Acknowledgments

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THE EFFECT OF GROWTH HORMONE ON THE UPTAKE OF NEUTRAL FAT BY THE LIVER *IN VITRO*

ABSTRACT

The effect of hypophyseal growth hormone on liver fat metabolism has been investigated in the perfused rat liver *in vitro*. It was observed that the uptake of neutral fat by the liver was accelerated either by the administration of the hormone to the animal several hours prior to sacrifice, or by the addition of the purified hormone to the perfusion fluid. These experiments suggest that growth hormone may act directly on hepatic tissue to alter the rate of uptake of lipid.

The rate of uptake of neutral fat by the perfused rat liver *in vitro* is markedly affected by the hormonal balance of the animal at the time the liver is removed (1). The administration of crude anterior pituitary extracts to the normal fed male rat, alloxan diabetes, or 48 hours of fasting resulted in a marked increase in the rate of lipid uptake by the livers. We wish to report here the stimulation of lipid uptake in the perfused liver induced either by the administration of anterior pituitary growth hormone to the animal before sacrifice or by the addition of the hormone to the perfusion fluid *in vitro*.

Neutral fat emulsions in Krebs-Henseleit bicarbonate buffer were perfused through isolated rat livers for 30 minutes at 37° C. Neutral fat uptake was measured by the decrease in turbidity or decrease of fatty acid esters in the perfusing medium. Bovine growth hormone (Lot M208), obtained from Armour and Co., and Somar A-Armour (Lot R50109), obtained from the Endocrinology Study Section of the National Institutes of Health, were employed in this study. The results obtained are shown in Table 1.

Growth hormone, when added directly to the perfusion medium, or given to the animal as a single injection 3–8 hours prior to removal of the liver, increased the rate of

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TABLE 1

Growth hormone administered	Uptake of neutral fat by liver Micromoles triglyceride/100 mg. liver/hour Mean ± S.E.M.	
	Emulsion #1* G.H.-M208	Emulsion #2† G.H.-R50109
(A) None	(25) 7.40 ± 0.35	(15) 3.79 ± 0.34
(B) 20 μg./ml. in perfusion fluid	(7) 13.45 ± 0.41	(9) 5.99 ± 0.71
(C) 1.0 mg./rat (3–8 hours before liver removal)	———	(15) 5.97 ± 0.47
(D) 1.0 mg./rat/day (2 days before liver removal)	(12) 8.02 ± 0.78	(10) 4.22 ± 0.68
Statistical Analysis	A vs. B t = 7.37 p = 0.0001	A vs. B t = 3.12 p = 0.0025
		A vs. C t = 3.71 p = 0.001
	A vs. D t = 0.76 p = 0.20	A vs. D t = 0.62 p = 0.25

The figures in parentheses indicate the number of animals. The livers used were obtained from 300 gm. adult male Sprague-Dawley rats fed Purina Laboratory Chow *ad libitum*. The animals were killed by decapitation and the livers were rapidly cannulated and removed. Livers were perfused via the vena cava. Growth hormone in 0.4 ml. of 0.9% NaCl was injected subcutaneously 3–8 hours prior to sacrifice, or daily for two days prior to removal of the liver. Cottonseed oil emulsions, prepared for intravenous human use, were diluted with a Krebs-Henseleit bicarbonate buffer, pH 7.4, such that Emulsion #1 contained oil 0.60, D-glucose 0.10, and the following agents for emulsification and stabilization: soybean phosphatides 0.02, sodium cholate 0.002, Ethofat C/15 (monofatty acid polyoxyethylene glycol ester) 0.02, Aldo 28 (glyceryl monostearate) 0.02, Tween 60 (sorbitan monostearate polyoxyethylene glycol ester) 0.02, Demal-14 (oleic acid polyglyceryl ester) 0.01, and Span 20 (sorbitan monolaurate) 0.05% (w/v) respectively. Emulsion #2 (Lipomul (Upjohn)) contained oil 0.45, D-glucose 0.12, phosphatides 0.036, and Pluronic F-68 (a polyoxyalkalene nonionic detergent) 0.009% (w/v) respectively. 50.0 ml. of emulsion, continuously oxygenated with 95% O_2–5% CO_2, was recirculated through a lucite-polyethylene perfusion apparatus. For the turbidometric analysis, 0.3–0.5 ml aliquots of the perfusate were diluted with 5.0 ml. of distilled H_2O, and the optical density at 700 millimicrons was determined in a Beckman DU spectrophotometer. Fatty acid esters were measured by hydroxamic acid formation (8). Turbidity and hydroxamate formation were directly proportional to emulsion concentration. The rates of fat uptake are expressed per 100 mg. of acetone-dried liver.

* Turbidometric assay.

† Hydroxamic acid assay.

uptake of fat by the isolated liver. When the hormone, however, was injected into fed or fasted rats at 48 and 24 hours before the liver was removed, no effect was observed under our experimental conditions. These data suggest that growth hormone has a direct stimulating effect on the process by which the liver takes up neutral fat. The observations are consistent with earlier studies demonstrating the production of fatty livers *in vivo* following the injection of anterior pituitary extracts and growth hormone to dogs (2), mice (3), and rats (4, 7). It is of particular interest that Greenbaum and McLean reported that the maximum increase in liver and blood lipids of rats occurs within the first 24 hours after receiving 0.5 mg. bovine growth hormone, and that, despite further daily dosage thereafter, blood and liver lipids returned to normal levels (7).

From the data reported here and elsewhere (1), it is apparent that certain hormones may regulate, in part, the rate of uptake of fat by the liver. This regulation may play an important role in the physiological adjustments during deprivation of food or insulin which increase fat combustion as a source of metabolic energy.

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THYROIDAL I^{131} UPTAKE IN FETAL SHEEP[1]

ABSTRACT

The I^{131} concentration of the fetal thyroid gland in sheep was found to increase exponentially during the second to the fifth month of life. The total I^{131} in the fetal thyroid one month before parturition approximated the daily dose of radioiodine administered to the dam. In the fifth month of gestation the I^{131} concentration in the thyroid gland of the fetus exceeded that of the dam's thyroid.

A previous paper (1) reported that uptake of I^{131} by the fetal thyroid gland of the sheep begins at about 50 days of gestation. This experiment was designed to determine the I^{131} concentration in fetal thyroid glands at semimonthly intervals during the remainder of the gestation period, about 95 days.

METHODS

Three groups of six adult ewes each, maintained on a diet low in stable iodine, were used in the experiment. One group (control) received no I^{131}; the other groups received, respectively, 0.15 and 5 μc. of I^{131} per day for more than 42 months. Cesarean section was performed on one animal of each group at 55, 70, 85, 100, 115, and 130 days of gestation. Autopsies were performed on the fetuses and the I^{131} concentration was determined in the various tissues and organs. Weight and size of the fetuses and of the principal body organs were recorded.

RESULTS AND DISCUSSION

Thirty fetuses were recovered from the eighteen ewes. The growth rate of the fetuses

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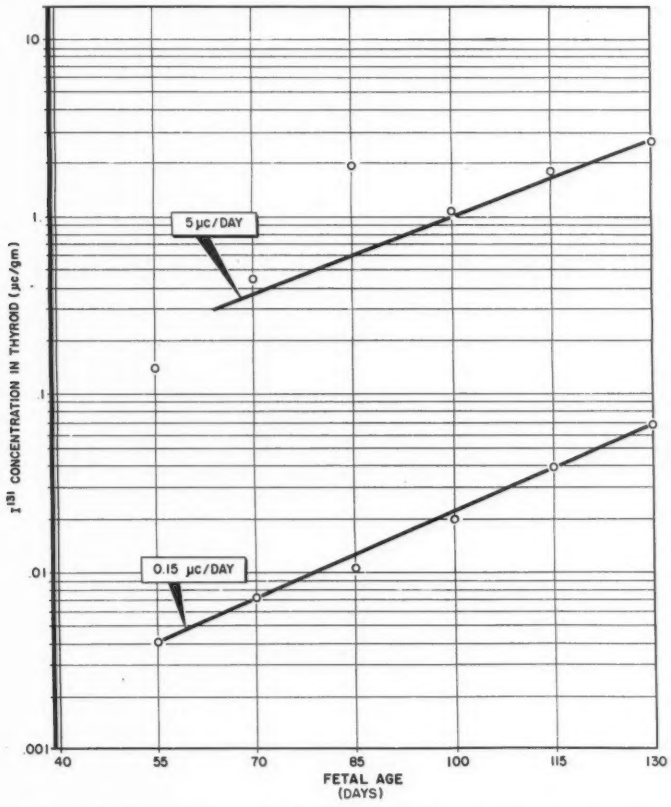

FIG. 1. Concentration of radioiodine (I^{131}) in fetal thyroid glands.

was slightly higher than that reported by Barcroft (2) and may be attributed to the high nutritional plane maintained in the experimental animals.

An exponential rise in thyroid concentration of I^{131} with increase of the fetal age was observed (Fig. 1). The high radioiodine concentration in the 85-day fetal thyroid gland of the 5 μc./day group (Fig. 1) was attributed to the relatively low concentration found in the damaged thyroid gland of the dam. The adult thyroid glands showed varying degrees of radiation damage (3, 4).

The fetal thyroid I^{131} concentration in relation to the daily maternal dose appears in Table 1.

TABLE 1. CONCENTRATION OF RADIOIODINE BY THE FETAL THYROID
(Expressed as percentage of the dam's daily dose)

Group feeding level	Days in gestation					
	55	70	85	100	115	130
5 μc./day	0.1*	1.1	12*	31*	58*	110*
0.15 μc./day	0.1*	1*	4*	32	79	94*

* Average of twins.

TABLE 2. CONCENTRATION OF I^{131} IN MATERNAL AND FETAL THYROID (μC./GM.)

Group feeding level		Fetal age at sacrifice					
		55	70	85	100	115	130
5 μc./day	Dam	0.8	3.0	0.8	1.1	1.0	2.6
	Offspring	0.14	0.5	2.0	0.8 1.5	1.8	2.6 2.8
0.15 μc./day	Dam	0.023	0.044	0.034	0.028	0.028	0.028
	Offspring	0.004 0.005	0.008 0.007	0.006 0.013	0.013 0.026	0.05 0.032	0.08 0.05

The total I^{131} in the fetal thyroid approximated the daily dose of radioiodine administered to the dam in advanced pregnancy.

Comparative concentrations of I^{131}/gm. of fetal and maternal thyroid glands are shown in Table 2. The concentration of radioiodine in the fetal thyroid was one to two times that of the dam's thyroid by 115 days gestation. The quantitative difference in thyroid avidity for radioiodine between the dam and the fetus in advanced pregnancy was observed in cows by Gorbman, *et al.* (5). The high I^{131} concentration factor exhibited by the fetus emphasizes the criticality of the fetal thyroid gland in assessing radioiodine toxicity.

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LOCALIZATION OF THE THYROXINE-BINDING PROTEIN OF SERUM BY STARCH GEL ELECTROPHORESIS[1]

ABSTRACT

The specific thyroxine-binding protein has been shown to migrate ahead of the serum albumin in a sharply localized band during starch gel electrophoresis, and to correspond in position to the first pre-albumin band.

A considerably greater purification of thyroxine-binding protein can be obtained by the method of starch gel electrophoresis than by conventional electrophoresis. The possibility is raised that the first pre-albumin band represents, in considerable part, the thyroxine binding protein.

Several techniques, including electrophoresis and chromatography have been used in attempts to isolate and characterize the thyroxine-binding protein (TBP) of human serum (1–5). It seemed possible that the method of starch gel electrophoresis, recently introduced by Smithies (6), might offer some advantage over the conventional methods of zone electrophoresis in the study of the TBP, because the separation of proteins in this medium depends not only on charge, but also partly on the size and molecular configuration of the proteins concerned. This is a report of preliminary observations of the electrophoretic mobility of TBP in a starch gel.

METHODS

Varying amounts of L-thyroxine (0.05 to 51 μg.) containing I^{131} labeled thyroxine[2] were shaken with 1.0 ml. of human serum in stoppered tubes for 16 hours at 2°. Electrophoresis was carried out in a starch gel (6). Because it was found that rapid widening of the resulting bands occurred due to diffusion, the electrophoretic conditions were designed to achieve maximum separation in minimum time. After electrophoresis, the starch gel was stained with amido black and cut into 2 mm. sections. These were transferred quantitatively into planchettes and the radioactivity measured. The radioactivity in each section of the starch gel was then compared to the position of the observed stained protein bands. In each case an aliquot of the serum used for electrophoresis was precipitated with trichloroacetic acid, washed and assayed for radioactivity. An aliquot of each serum was extracted with n-butanol and a paper chromatogram prepared using the acid solvent described by Roche (7). In all instances between 80 and 95% of the activity was precipitated by the addition of trichloroacetic acid. Chromatographic analysis showed that 80 to 90% of the radioactivity had the same mobility as thyroxine, and the remainder that of iodide standards.

RESULTS

The localization of the radioactivity after electrophoresis is illustrated in Figure 1. In all experiments radioactivity was present in the albumin and also in the leading pre-albumin band. No significant radioactivity was associated with a narrow band between the albumin and the leading band, or with any other protein fraction.

Another sample of serum from the same subject was equilibrated and subjected to conventional starch zone electrophoresis in a barbital buffer, pH 8.6, $\tau/2 = 0.1$. After separation, the starch block was cut into sections and the protein eluted by displacement filtration. The radioactivity was localized to a zone intermediate in position between the α_1 and α_2 proteins, and also found in association with the albumin fraction. The protein in the segments containing the maximum radioactivity was concentrated and re-run separately in a starch gel. Whereas the isolated albumin, when re-run, migrated as a single broad band, the material separated from the α_1-α_2 area was found to contain

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at least eight protein bands after electrophoresis in the starch gel. Moreover, the entire radioactivity was associated with one protein band which corresponded in position to the leading (first) pre-albumin band found by starch gel electrophoresis of the original whole serum. Thus a considerable increase in specific activity of the thyroxine-binding protein had been achieved.

In some experiments, after electrophoresis, radioautographs of the starch gel were

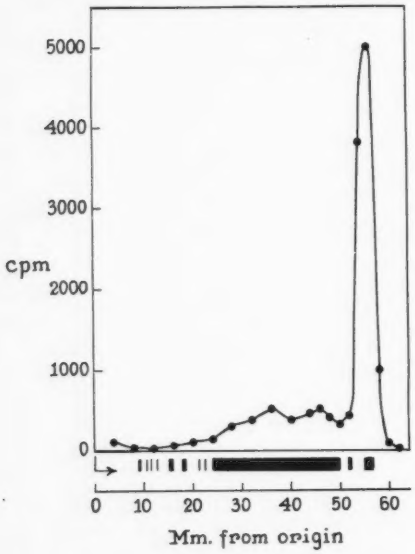

FIG. 1. Starch gel electrophoresis of 1 ml. normal human serum equilibrated with 0.40 μg. I^{131} labelled l-thyroxine. The solid bars indicate the positions of the protein components. The curve illustrates the radioactivity associated with each segment of the starch gel. Approximately 64% of the total serum radioactivity was present in the first pre-albumin band.

made. Although the localization of the radioactivity in the pre-albumin region was clearly demonstrable, the time necessary to develop the autographs was such that diffusion of the protein bands occurred and the TBP could not be localized with the precision obtained by the direct counting technique.

DISCUSSION

The electrophoretic separation of proteins in a starch gel depends upon the character of the gel used, the nature of the buffers employed, and on other imperfectly understood factors. Under the conditions of these experiments, two distinct protein bands migrated towards the cathode more rapidly than albumin. The band which migrated most rapidly, the first pre-albumin band, always contained about 60% of the radioactivity when 0.05 to 2.0 μg./ml. I^{131} labelled thyroxine was added to the serum.[3] The first pre-albumin band is thus partly or wholly composed of TBP.

The presumption that the TBP is one of the group of small α_2 glycoproteins described by Schmid (8) could be reasonably supported by the present observations, because the small size of the TBP might permit its rapid migration in a starch gel. However, it is not excluded that other factors, such as the pH and the ionic strength of the buffer used

[3] When between 6 and 51 μg. thyroxine was added/ml. serum about 2.2 μg. was associated with TBP and the rest with albumin. Thus TBP was able to combine with approximately 2.2 μg. thyroxine/ml. serum, a value about ten times greater than that reported after reverse flow paper electrophoresis in a barbital buffer (4). Further work will be required to clarify the reason for this apparent difference.

may play an equally important role. The well defined separation of TBP from other major serum proteins obtained by starch gel electrophoresis suggests that the method may be useful in further studies of this protein. Although thus far the technique has been used primarily in a qualitative fashion, preliminary experiments indicate that it may prove of value in isolating serum proteins on a more preparative scale.

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THE UTERINE GROWTH-STIMULATING ACTIVITIES OF 17α-ETHYNYL-17-HYDROXY-5(10)-ESTREN-3-ONE (NORETHYNODREL) AND 17α-ETHYNYL-19-NORTESTOSTERONE[1]

ABSTRACT

Despite structural similarity and certain common biological properties, the uterine growth-stimulating activities of Norethynodrel and 17α-ethynyl-19-nortestosterone were entirely different when dose-response curves of uterine weight were compared. These differences obtained whether the material was administered parenterally or intragastrically. In this test Norethynodrel acted like an estrogen (i.e., it had a steep dose-response curve slope), whereas 17α-ethynyl-19-nortestosterone behaved like an impeded estrogen, a testoid or a progestin (shallow slope). 17α-Ethynyl-19-nortestosterone was an antagonist of both Norethynodrel and estrone, whereas, Norethynodrel had no effect on estrone-induced uterine growth. Again these relationships were qualitatively similar for intragastric and subcutaneous administration. Qualitatively each compound appeared to maintain its specific integrity regardless of route of administration.

17α-Ethynyl-17-hydroxy-5(10)-estren-3-one (Norethynodrel) and 17α-ethynyl-19-

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nortestosterone are similar chemical entities, differing only in the position of the double bond in the A ring. In addition to structural similarities, the substances have certain biological properties in common (1, 2). These data suggested that the progestational activities of both compounds could have resulted from a single active entity. This suggestion was strengthened by the fact that both were reported as highly active progestational agents on oral administration (2, 3), but that neither was particularly potent when instilled directly into the uterus (2, 4). Pincus, *et al.* (1) have reported that Norethynodrel had a dose-response curve with a steep slope in a mouse uterine growth assay, whereas pure 17α-ethynyl-19-nortestosterone had a shallow slope, similar to those of the impeded estrogens (5, 6). These studies have now been extended to intragastric

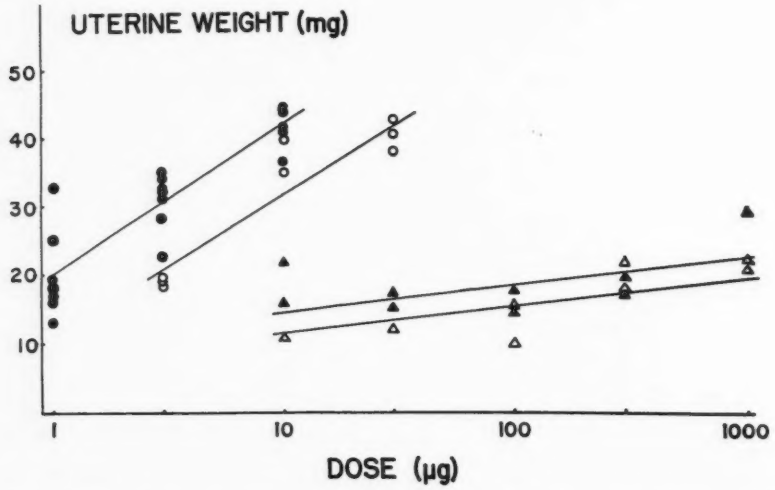

FIG. 1. The uterine growth-stimulating effects of Norethynodrel (dots) and 17α-ethynyl-19-nortestosterone (triangles). Solid symbols, subcutaneous administration; hollow symbols, intragastric route. Each point represents the mean of 8–10 mice. Regression lines calculated by method of least squares. Simultaneous oil control groups averaged about 12 mg. in uterine weight.

administration, employing our modification of the Rubin test (6), which is essentially similar to that used by Pincus and his colleagues.

Comparison of dose-response curves for intragastric and subcutaneous Norethynodrel and 17α-ethynyl-19-nortestosterone. The dose-response curve for intragastrically (IG) administered Norethynodrel was remarkably similar in slope to the curve for the same material injected subcutaneously (SC) (Fig. 1). The actual values for the slopes were:

Route	*b*
IG	21.30
SC	21.94

For comparison, recent data on subcutaneously administered estrone produced a curve with a slope of 24. Intragastrically, Norethynodrel was estimated to be about 35% as potent as it was subcutaneously. 17α-Ethynyl-19-nortestosterone appeared to be less potent by the intragastric route, although an accurate estimate is difficult because of

TABLE 1. THE INHIBITION OF NORETHYNODREL-INDUCED UTERINE GROWTH BY 17α-ETHYNYL-19-NORTESTOSTERONE

Dose (μg.) Norethynodrel	Dose (μg.) 17α-ethynyl-19-nortestosterone	Uterine weight (mg.)*
Subcutaneous administration		
4	3	31.9
4	3	33.0
4	10	26.6
4	10	27.7
4	30	24.8
4	30	25.7
4	100	19.4
4	100	19.0
4	300	21.3
4	300	23.6
uterine weight = 33.67 − 5.63 log dose (P < 0.01 that b = 0)		
Intragastric administration		
10	10	32.0
10	10	29.3
10	30	25.9
10	30	24.9
10	30	24.0
10	100	22.4
10	100	20.8
10	100	19.4
10	300	18.7
10	1000	20.3
uterine weight = 34.43 − 5.95 log dose (P < 0.01 that b = 0)		

* Each value is the mean of 8–10 mice.

the shallow slopes of the curves. With this material there were slight differences in slopes of the dose-response curves:

Route	$b \pm S.E.$
IG	6.25 ± 2.56
SC	3.53 ± 2.18

These differences were not statistically significant ($t = 0.810$, d.f. = 15, $P > 0.40$) so a pooled slope was employed ($b = 4.25$).

Uterine growth effects of mixtures of Norethynodrel and 17α-ethynyl-19-nortestosterone. Over the past several years in this laboratory we have come to consider that compounds that have steep dose-response curve slopes are true estrogens, i.e., produce vaginal cornification in spayed rats, whereas those with impeded slopes may be estrogens, androgens, or progestins. Our studies have also shown that certain nonestrogenic uterine growth-stimulators will antagonize the uterine growth-stimulating effects of the estrogens (7). The impeded estrogens have also been implicated as estrogen antagonists (5, 8). Therefore, it became of interest to learn whether 17α-ethynyl-19-nortestosterone would inhibit the uterine growth response to Norethynodrel.

Experiments, which employed essentially the design described previously (7), were carried out. Varying amounts of 17α-ethynyl-19-nortestosterone were administered, mixed with a constant dose of Norethynodrel. In the first study 4 μg. of Norethynodrel were used and the materials were administered subcutaneously. In a similar experiment, 10 μg. of Norethynodrel were employed; administration was intragastric. In both experiments 17α-ethynyl-19-nortestosterone was found to be an inhibitor of the uterine growth promoted by Norethynodrel (Table 1).

TABLE 2. THE INHIBITION OF UTERINE GROWTH INDUCED BY THE SUBCUTANEOUS ADMINISTRATION OF 0.3 μg. OF ESTRONE BY SUBCUTANEOUSLY AND INTRAGASTRICALLY ADMINISTERED 17α-ETHYNYL-19-NORTESTOSTERONE

Dose (μg.)	Uterine weight (mg.)*
Subcutaneous	
3	30.4
10	24.7
10	19.2
30	16.8
30	21.8
100	17.2
uterine weight = 31.75 − 8.14 log dose ($P < 0.01$ that $b = 0$)	
Intragastric	
3	34.3
10	30.7
30	26.1
100	24.2
300	23.9
uterine weight = 35.97 − 5.47 log dose ($P < 0.02$ that $b = 0$)	

* Each value is the mean of 8–10 mice.

The effects of Norethynodrel and 17α-ethynyl-19-nortestosterone on estrone-induced uterine growth. The inhibition of Norethynodrel-induced uterine growth by 17α-ethynyl-19-nortestosterone suggested that a similar study employing a natural estrogen was warranted. Therefore, a series of experiments have compared the effects of 17α-ethynyl-19-nortestosterone and Norethynodrel for their effects on estrone-induced uterine growth. In all studies estrone was administered subcutaneously at a dose of 0.3 μg. Test compounds were administered subcutaneously, in which case they were mixed with the estrone, and intragastrically in separate experiments. 17α-Ethynyl-19-nortestosterone proved to be an inhibitor of estrone-induced uterine growth both subcutaneously and intragastrically (Table 2). Norethynodrel, had no effect upon this growth by either route (Table 3). It seems noteworthy that dose levels of Norethynodrel that alone pro-

TABLE 3. FAILURE OF SUBCUTANEOUSLY AND INTRAGASTRICALLY ADMINISTERED NORENTHYNODREL TO AFFECT THE UTERINE GROWTH STIMULATED BY SUBCUTANEOUSLY ADMINISTERED ESTRONE (0.3 μg.)

Dose (μg.)	Uterine weight (mg.)*
Subcutaneous	
3	44.0
3	37.5
10	39.0
10	44.4
30	44.8
30	42.0
100	37.4
100	39.2
uterine weight = 42.46 − 1.15 log dose $P \cong 0.60$ that $b = 0$)	
Intragastric	
3	34.9
10	43.9
30	33.6
100	34.7
300	36.8
uterine weight = 38.31 − 1.03 log dose ($P > 0.70$ that $b = 0$)	

* Each value is the mean of 8–10 mice.

duced marked growth of the uterus had no inhibitory or additive effect upon uteri submaximally stimulated with estrone.

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ANTI-ANDROGENIC ACTIVITY OF A SYNETHETIC PHENANTHRENE

ABSTRACT

A new synthetic phenanthrene derivative, 2-acetyl-7-oxo-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydrophenanthrene, has been found to have anti-androgenic and anti-myotrophic activity. The phenanthrene derivative antagonized the hypertrophy of the seminal vesicles, ventral prostate and levator ani muscle induced by testosterone propionate in the immature, castrated rat. When administered alone in large doses there was no significant effect on the seminal vesicle, ventral prostate or levator ani muscle of either the immature, intact or the immature, castrated rat. There was no estrogenic or anti-estrogenic activity in female rats. In immature male or female rats, there was no significant change in body weight, or weights of adrenals, thymus or thyroid glands.

Testosterone propionate possesses high myotrophic anabolic activity as well as high androgenic activity (Eisenberg and Gordon (1)). The myotrophic activity was measured by the increase in weight of the levator ani muscle while the androgenic activity was measured by the increase in weight of the seminal vesicle. Marked differential effects on these end organs have been observed for various steroids. For instance, 19-nor testosterone had high myotrophic and low androgenic activity whereas androsterone had the reverse effect (Hershberger, Shipley and Meyer (2)). Certain antagonists to the myotrophic and androgenic activity of testosterone were observed by Pincus and Dorfman (3). Hydrocortisone and corticosterone inhibited when administered at 20 times the dose level of testosterone. It was stated that progesterone was a particularly strong inhibitor of the androgenic activity and it also inhibited the myotrophic activity of testosterone. Various analogs of progesterone failed to antagonize the effects of testosterone.

Received December 23, 1957.

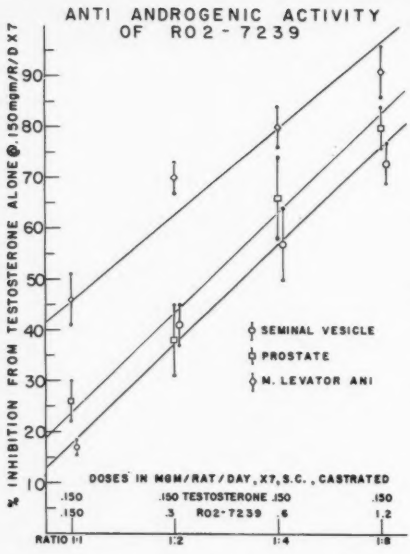
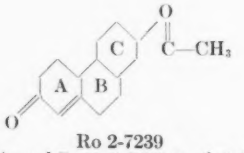
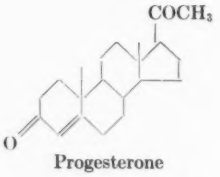


FIG. 1. Anti-androgenic activity of Ro 2-7239. Log of the dose of Ro 2-7239, the constant dose of testosterone propionate and the ratio of the doses of Ro 2-7239 to testosterone are plotted on the abcissa. The per cent inhibition of testosterone hypertrophy of the seminal vesicle, prostate or levator ani muscle is plotted on the ordinate. The points through which the straight lines are drawn represent the average and standard errors of 5 rats per group

This paper reports the antagonistic effects to testosterone of a synthetic phenanthrene derivative which had some superficial structural resemblance to progesterone. It has both anti-androgenic and anti-myotrophic activity. The structure is as follows:



Ro 2-7239
2-Acetyl-7-oxo-1,2,3,4,4a,4b,5,6,7,
9,10,10a-dodecahydro-phenanthrene



Progesterone

The compound was synthesized by Drs. M. W. Goldberg and W. E. Scott, in the Chemical Research Labs. of Hoffmann-La Roche, Inc.

METHODS

Charles River rats of 70 gm. weight were castrated under light ether anesthesia. The rats were separated into groups of 5 rats. Beginning on the first day after castration, the rats were injected subcutaneously (S.C.) daily for 7 days with 0.15 mg. testosterone propionate dissolved in sesame oil.

Ro 2-7239, dissolved in sesame oil, was injected, S.C., in doses of 0.15, 0.3, 0.6, and 1.2 mg. into groups of the rats receiving testosterone. The ratio of androgen to anti-androgen in the various groups was thus: 1:0, 1:1, 1:2, 1:4, and 1:8. A group of 5 control rats received saline and a group of 5 rats received Ro 2-7239 alone at a dose of 1.2 mg./rat. The rats were sacrificed 24 hours after the last dose and weights of the seminal vesicles, prostate and levator ani muscle were obtained. For measuring estrogenic activity, weanling female rats were injected S.C. daily for 7 days with Ro 2-7239 alone and in combination with estradiol benzoate. The drugs were dissolved in sesame oil and control rats were injected with sesame oil alone. Testosterone in the dose of 0.15 mg./rat/day for 7 days caused an increase of 565% in the weight of the seminal vesicles, 365% in the weight of the prostate and 105% in the weight of the levator ani muscle.

RESULTS

Figure 1 illustrates the inhibitory effects of Ro 2-7239 on the increase in size of the

TABLE 1. 7 DAY TEST FOR ESTROGENIC ACTIVITY IN RATS
(Average and standard errors of 5 rats per group)

Group and drug	Final body, gm.	Uterus, mgm./100 gm.	Adrenals, mgm./100 gm.	Thymus, mgm./100 gm.
Control .1 cc. Sesame Oil/rat/day	95.4 ±19.8	36.4± 5.7	36.5 ±1.3	257.9 ±43.2
Estradiol Benzoate 12.5 μg./rat/day	102.0 ±6.7	166.4±10.6	38.7 ±1.2	231.8 ±11.4
Estradiol Benzoate 25 μg./rat/day	92.2 ±5.5	191.1±38.8	42.8 ±5.4	176.5 ±20.8
E.B.* 12.5 μg. + Ro 2-7239 .5 mg/rat/day	95.8 ±4.6	191.2±36.5	36.9 ±1.2	199.2 ±19.3
E.B.* 12.5 μg. + Ro 2-7239 1.0 gm./rat/day	97.4 ±3.0	204.8±24.3	36.0 ±1.3	235.2 ±22.3
Ro 2-7239 .5 mg./rat/day	116.7 ±3.2	29.5± 2.8	34.9 ±4.7	314.3 ±31.4
Ro 2-7239 1.0 mg./rat/day	99.8 ±4.9	32.6± 3.3	33.8 ±2.8	296.9 ±21.4

* E.B. = estradiol benzoate.

seminal vesicles, prostate and levator ani muscle induced by testosterone propionate. It will be noted that in the 1/1 ratio of testosterone and Ro 2-7239, the seminal vesicle hypertrophy was inhibited 46%, the prostate hypertrophy was inhibited 26% and the levator muscle was inhibited by 18%. Increasing doses of the inhibitor had greater antagonistic powers. The degree of inhibition was linearly related to the log of the dose since the plot of per cent inhibition and the log of the dose gave straight lines. The results indicate that there is competitive antagonism of Ro 2-7239 and testosterone at the end organs.

In control experiments Ro 2-7239 had no significant effect on the weight of the seminal vesicles, prostate or levator ani when given alone in doses up to 10.0 mg./rat S.C. for 4 days to the weanling male rats.

No estrogenic or anti-estrogenic activity could be demonstrated for the compound. The results in Table 1 show that doses of 0.5 and 4.0 mg./rat/day, S.C., for 7 days had no significant effect on the weights of the uterus, and no antagonistic effect to the hypertrophy of the uterus induced by estradiol benzoate. There were no significant changes in the body weights, adrenal weights, or thymus weights.

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EFFECTIVENESS OF DHE IN BLOCKING EPINEPHRINE-INDUCED HYPERGLYCEMIA IN RABBITS AND BULLFROGS[1]

ABSTRACT

The effectiveness of dihydroergotamine (DHE) in blocking epinephrine-induced hyperglycemia in rabbits was confirmed, although a higher dosage of DHE was required than used by Ellis and associates. Only at a dosage of 0.4 mg/kg. body weight was DHE completely effective and of practical value in distinguishing hyperglycemias induced by epinephrine and glucagon. DHE imposed its greatest inhibition within 10–15 minutes following intravenous injection. Pentobarbital (Nembutal) anesthesia abolished a significant part of the blocking activity of DHE in rabbits. DHE had no discernible glycogenolytic-blocking activity in bullfrogs.

Ellis, Anderson, and Collins (1) have presented data indicating that dihydroergotamine (DHE) effectively blocks the hyperglycemic activity of epinephrine but does not interfere with the capacity of glucagon to raise the blood sugar level in intact rabbits. In view of the seemingly general acceptance of these data as a working formula in many laboratories (attested to by numerous citations in recent literature), we have felt it important to report our experience with the use of DHE. Our results vary from those of Ellis *et al.*, and could have widespread bearing on much glucagon research now in progress. To our knowledge, information on the relationship of pentobarbital (Nembutal) to the blocking activity of DHE has not been presented previously and therefore constitutes an important factor to be reckoned with in laboratories where this manner of anesthetization of experimental animals is standard procedure. Data on the response of the bullfrog, *Rana catesbiana*, to DHE and epinephrine are included for comparative purposes.

MATERIALS AND METHODS

Rabbits were fasted for 20–22 hours before use. Actually an 8-hour fasting period proved to give equally uniform results, but the longer interval was usually more convenient. Blood was obtained from marginal ear veins, from the central ear artery, or by ventricle puncture, and the site of withdrawal did not influence sugar values. Injections in rabbits were intravenous. Frogs (80–700 gm.), obtained from collectors in Wisconsin and Louisiana, were posteriorly pithed and the pericardial area exposed. After heparinization, blood samples were taken from the truncus arteriosus. Injections in frogs were intra-arterial. Blood glucose values were determined according to the method of Nelson (2), except that NaOH was used in place of $Ba(OH)_2$ in making protein-free filtrates.

Rabbits were divided into 8 groups of 5 animals each, and the treatments accorded them are shown in Table 1. When DHE was used the initial blood sample was taken, and epinephrine given, 25–30 minutes after DHE. When pentobarbital and no DHE was administered, the initial sample was withdrawn within 2 minutes after the animal was anesthetized. Final samples (Groups I–VII) were taken 25–30 minutes after giving epinephrine; in group VIII, 25–30 minutes following initial sample. Frog groups also consisted of 5 animals each, and the timing of blood samples was the same as for rabbits.

RESULTS

It is apparent from Table 1 that the blocking effect of 0.2 mg./kg. DHE (Group I, the dosage used by Ellis *et al.*) is incomplete in unanesthetized rabbits, and the variability is high. Doubling the dosage of DHE (Group III) produced effective blockade with less variability, particularly if the dosage of epinephrine is reduced to 0.015 mg./kg. (Group IV), an amount well within the normal range for optimal hyperglycemic response.

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[1] Supported by research grant A-1280 from the National Institutes of Health, Public Health Service.

TABLE 1. EFFECTS OF DHE AND PENTOBARBITAL ON EPINEPHRINE-INDUCED HYPERGLYCEMIA IN RABBITS AND BULLFROGS

Group	Dosage, mg./kg.			Avg. blood sugar (mg.%) ±S.E.[3]		Average % change
	DHE[1]	Epinephrine[2]	Pento-barbital	Initial	Final	
I	0.2	0.03	0	92 ± 4	106 ± 7	16 ± 4
II	0.2	0.03	25	104 ± 4	132 ± 12	30 ± 9
III	0.4	0.03	0	98 ± 6	105 ± 8	7 ± 3
IV	0.4	0.015	0	100 ± 4	103 ± 5	4 ± 2
V	0.4	0.015	25	79 ± 4	93 ± 6	17 ± 6
VI	0.0	0.03	0	84 ± 4	165 ± 12	94 ± 6
VII	0.0	0.03	25	102 ± 4	225 ± 42	116 ± 31
VIII	0.0	0.0	25	107 ± 4	110 ± 8	2 ± 4
Frog A	0.4	0.03	0	2 ± 1	28 ± 3	
Frog B	0.0	0.03	0	4 ± 2	28 ± 6	

[1] Dihydroergotamine methanesulfonate Sandoz.
[2] Adrenalin chloride Parke, Davis Co.
[3] Standard Error.

Particular attention should be called to 3 aspects of pentobarbital activity: (1) It partially negates the effectiveness of DHE (Groups II and V), (2) It does not significantly enhance the hyperglycemic activity of epinephrine (Group VII) although variability is increased, and (3) It exhibits no hyperglycemic activity when administered separately (Group VIII).

The low initial blood glucose values for frogs have been reported previously (3) and are without an immediate explanation. Epinephrine is effective in raising this level, but it is interesting that DHE showed no sign of blocking epinephrine's action in the frog at a dosage found effective for rabbits.

DISCUSSION

The student of glucagon has a valuable tool in the proper use of DHE, for he can effectively eliminate the action of contaminating epinephrine in tissue extracts being tested for glucagon activity. Presumably extracts of pancreas or gastrointestinal tract would contain only trace amounts of epinephrine or compounds of similar activity, but this possibility, however slight, must be avoided nevertheless. We respectfully maintain that concentrations of DHE used by Ellis *et al.* are insufficient to completely eliminate the activity of epinephrine, and that higher dosages are required. Whereas Ellis *et al.* obtained a mean difference of +6 mg.% between initial and final blood samples of rabbits given 0.2 mg./kg. DHE and 0.03 mg./kg. epinephrine, our results showed an average difference of +16 mg.%. If 0.4 mg./kg. is used, however, this difference is cut to less than half. If the dosage of epinephrine is reduced to 0.015 mg./kg., this higher dosage of DHE is almost completely effective in inhibiting hyperglycemia.

In our experience, the peak of DHE inhibition of epinephrine activity did not come at 30 minutes, as Ellis has indicated, but rather between 10 and 15 minutes following injection. For example, a repeat (6 animals) of the experimental procedure used in Group III (Table 1), allowing DHE to act for only 15 minutes instead of 30, gave an average % change of only 4 ± 1. Further improvement in the results for Group IV could also be obtained by allowing a shorter interval following DHE injection.

Our results indicate that DHE could not be used reliably in animals anesthetized with pentobarbital for the anesthetic noticeably reverses a large portion of the DHE blockade. The mechanism for pentobarbital interference with DHE activity is not known, but a similar relationship has been recognized previously in connection with anesthesia and the hypertensive activity of crude ergot extract (4).

In assaying extracts of tissues for hyperglycemic activity in the bullfrog, it is necessary to rule out the presence of epinephrine by other tests (*e.g.*, frog melanophore reaction) since DHE fails to impose any block whatsoever on epinephrine-induced glycogenolysis in frog liver. In general, however, the use of bullfrogs for assay of hyperglycemic agents has many advantages over the more conventional use of rabbits.

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The Endocrine Society

THE 1958 ANNUAL MEETING

The Fortieth Annual Meeting of The Endocrine Society will be held in the St. Francis Hotel, San Francisco, California, Thursday, Friday and Saturday, *June 19, 20, and 21, 1958.*

The Committee on Local Arrangements—Dr. Roberto Escamilla, Chairman; Dr. Donald E. Bernstein, Dr. Peter H. Forsham, Dr. Minnie B. Goldberg, Dr. Gilbert S. Gordan, Dr. Francis S. Greenspan, Dr. C. H. Li, as members of the committee.

All Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily in the Colonial Ballroom, and in addition there will be simultaneous afternoon sessions in the Italian Room. The annual dinner is scheduled for Friday, June 20 at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All reservations for rooms must be made through the AMA Housing Bureau, Room 300, 61 Grove Street, San Francisco 2, California. If possible, official application forms as published in the Journal of the American Medical Association should be used. Arrival and departure dates must be stated. All requests must be accompanied by check or money order (not cash) made payable to "The AMA Housing Bureau" in the amount of $10.00 per room, which will be credited to your hotel account. Be sure to state your first, second and third choice of hotels. It is important to make reservations immediately in order to avoid disappointment.

Final program, membership card and advance registration forms will be sent on May 15, 1958 to members whose current dues have been paid.

PROGRAM

THURSDAY, JUNE 19, 1958

8:30 A.M.—Registration—Mezzanine, St. Francis Hotel

Final session of the American Goiter Association program will be held in the Colonial Room. Those registered for The Endocrine Society meeting will be admitted to this program.

I. 9:00 A.M.—ENDOCRINE AND METABOLIC INTERRELATIONSHIPS

Italian Room, St. Francis Hotel

Chairmen: *Leon Hellman and Dwight Ingle*

1. AN APPRAISAL OF THE EXTRA-ADRENAL METABOLIC ACTIONS OF CORTICOTROPIN. *Frank L. Engel.*

2. Plasma Corticosterone Following Transplantation of the Adrenal Gland to the Portal Circulation.
   Donald E. Bernstein, George R. Biskind, and *Nicolas Zenker* (by invitation).
3. Cortisol Suppression of Endotoxin Induced Serum Transaminase Elevation.
   James C. Melby, Richard H. Egdahl (by invitation), and *Wesley W. Spink.*
4. Some Obervations upon Adrenal Cortical Function During Pregnancy.
   Nicholas P. Christy, W. E. L. Gordon (by invitation), *Donald Longson* (by invitation), *Eleanor Z. Wallace,* and *Joseph W. Jailer.*
5. Adrenocortical Hyperfunction in "Idiopathic Hirsutism and the Stein-Leventhal Syndrome.
   T. F. Gallagher, Attaliah Kappas, Leon Hellman, M. B. Lipsett, O. H. Pearson, and *C. D. West.*
6. 17-Ketosteroid Fractionation in Virilism.
   Herman E. Carr, Jr. (by invitation), *William J. Reddy,* and *George W. Thorn.*

INTERMISSION

7. Water Loading Test in Hypoadrenocorticism and Hypothyroidism.
   Arnold M. Moses (by invitation), *J. Lester Gabrilove,* and *Louis J. Soffer.*
8. Body Composition and in Vitro Synthesis of Lipids by Adipose Tissue of 11-Dehydrocorticosterone Treated Mice.
   Guy Hollifield, and *William Parson.*
9. Absence of Direct Adrenal Cortical Effect upon Lipogenesis by Rat Adipose Tissue.
   Bernhard G. Jeanrenaud (by invitation), and *Albert E. Renold.*
10. An Adrenal Effect on the Production of Nephrotic Hyperlipemia.
    Peter K. Bally (by invitation), and *Luis Dominge* (by invitation), (introduced by Kendall Emerson, Jr.).
11. Blocking the Renal Electrolyte Effects of Mineralocorticoids with Synthetic Steroidal Lactones.
    C. M. Kagawa (by invitation), *J. A. Cella* (by invitation), and *C. G. Van Arman* (by invitation), (introduced by Francis J. Saunders).
12. Pharmacologic Inhibition of Steroid Oxygenation in the 11 Position in Man and Dog.
    John S. Jenkins (by invitation), *Lillian Pothier* (by invitation), *J. W. Meakin* (by invitation) and *Don H. Nelson.*

II. 2:00 P.M.—THYROID AND PARATHYROID

Colonial Room—St. Francis Hotel

THOSE WHO HAVE REGISTERED WITH THE AMERICAN GOITER ASSOCIATION MEETING CAN ATTEND THIS SESSION WITHOUT REGISTERING WITH THE ENDOCRINE SOCIETY MEETING

Chairmen: *J. E. Rall and Sidney H. Ingbar*

13. The Failure of Acetyl-TSH or Pituitary-Inhibitor to Alter the Thyroid Slice Response to TSH.
    John L. Bakke, and *Nancy Lawrence.*
14. The Effect of Dinitrophenol on Thyroid Function in Rats with Hypothalamic Lesions.
    Seymour Reichlin.
15. The Effect of Hypophysectomy and of TSH on the Mouse Submaxillary Iodide Trap.
    Alvin Taurog, G. D. Potter (by invitation), and *I. L. Chaikoff.*
16. Demonstration of Antithyroid Activity Following Ingestion of a Naturally-Occurring Thioglycoside.
    J. M. Deeney (by invitation), and *Monte A. Greer.*

17. The Role of Thyroxine-Binding Interactions in the Thyroidal Release of Hormone.
J. Thomas Dowling (by invitation), *Sidney H. Ingbar*, and *Norbert Freinkel*.
18. The Metabolism of S^{35}- and C^{14}-Labeled Thiourea by Thyroid Cell Particulate Fractions.
F. Maloof (introduced by J. B. Stanbury).

INTERMISSION

19. Steroid Hormone Metabolism in Myxedema.
H. Leon Bradlow, Leon Hellman, B. Zumoff, and *T. F. Gallagher*.
20. Steroid Metabolism in the Thyroxin Treated Rat.
Joseph S. McGuire, and *Gordon M. Tomkins* (introduced by J. E. Rall).
21. Alteration in Tubular Reabsorption of Phosphorus by Triiodothyronine.
Milton E. Rubini, William R. Beisel (by invitation), *Harold S. Ranks* (by invitation), and *William B. Blythe* (by invitation).
22. Comparison of Intravenous Strontium and Calcium Loads.
D. Werdegar, B. Clarkson, and *M. Horwith* (introduced by Eugene J. Cohen).
23. Calcium Turnover in Man in Various Disease States.
E. Eisenberg (by invitation), *G. S. Gordon, R. Fraser* (by invitation), and *K. Ibbertson* (by invitation).
24. The Effects of Hypoparathyrodism, Acetazolamide, Parathormone, Dihydrotachysterol, and Vitamin D on Magnesium Metabolism in Man.
Leonard P. Eliel, William O. Smith (by invitation), *Carl Thomsen* (by invitation), and *Josephine Hawrylko* (by invitation).

III. 2:00 P.M.—ADRENAL AND GONADS

Italian Room, St. Francis Hotel

Chairmen: *J. W. Jailer and C. D. West*

25. Inhibitors of Adrenal Cortical Secretion.
Robert Gaunt, J. J. Chart, Nancy Howie (by invitation), *Betty Sullivan* (by invitation), and *H. Sheppard* (by invitation).
26. The Determination of Non-Protein Bound Plasma Hydrocortisone by Ultrafiltration.
Ivor H. Mills (by invitation), *Harold P. Schedl* (by invitation), *Diana Reed* (by invitation), and *Frederic C. Bartter*.
27. Metabolism of 4-C^{14}-Progesterone by Human Adrenal Microsomes.
W. R. Slaunwhite, Jr., and *A. A. Sandberg*.
28. Distribution of 16-C^{14}-Estrone Between Protein Fractions of Plasma and Red Blood Cells of Man.
Claude J. Migeon, Ono L. Lescure (by invitation), and *Harry N. Antoniades* (by invitation).
29. Identification of a Pregnancy Plasma Adrenotropin as Chorionic Gonadotropin.
Jonathan T. Lanman, and *Jenny Dinerstein* (by invitation).
30. Treatment of Ovarian Dysfunction with Small Doses of Cortisone or Hydrocortisone.
W. McK. Jefferies, and *R. P. Levy*.

INTERMISSION

31. Progestational Activity of Certain 19-NOR Steroids and a Progesterone Derivative.
Tamotsu Miyake.

32. 17-Ketosteroids of Meconium.
Nai-Hsuan Chang Shen, Faith Ellen Francis and *Ralph A. Kinsella, Jr.* (introduced by Edward A. Doisy).
33. Effect of Norethandrolone on 17-Ketosteroid Excretion in Prostatic Cancer Patients.
Herbert Prendlen and *Bertram S. Winkler* (introduced by William Money).
34. Inherent Estrogenicity of the Orally Active Progestational Steroids, Norethisterone and Norethynodrel.
C. Alvin Paulsen, Robert B. Leach, Norman Goldstone (by invitation), *John Lanman* (by invitation), and *William O. Maddock.*
35. Effect of *in vivo* Administration of Estrogens on Rat Adrenal Corticoid Secretion *in vitro.*
Kenneth W. McKerns, and *E. Kaleita* (by invitation).
36. Coexistence of Adrenal Virilizing Hyperplasia and Interstitial Cell Tumors in an Adult Male.
Stanley Newman, Gerold H. Mahaffey (by invitation), and *Vincent C. Di Raimondo.*

FRIDAY, JUNE 20, 1958

8:30 A.M.—Registration—Mezzanine, St. Francis Hotel

IV. 9:00 A.M.—PITUITARY AND HYPOTHALAMUS

Colonial Room, St. Francis Hotel

Chairmen: *A. E. Wilhelmi and Leslie L. Bennett*

37. A Simplified Method for the Isolation of Alpha and Beta Melanocyte Stimulating Hormones.
Sanford L. Steelman.
38. The Measurement of Serum Sulfation Factor as an Index of Somatotrophin Activity in Man.
William H. Daughaday, William D. Salmon (by invitation) and *France Alexander* (by invitation).
39. Assay of Growth Hormone for Activity in Man.
M. S. Raben, and *C. H. Hollenberg* (by invitation).
40. Human Growth Hormone: Prolonged Administration of Moderate Doses in Three Patients with Panhypopituitarism.
Philip H. Henneman, Marc Moldawer, Elvin L. Carroll (by invitation), *Mary Mitchell* (by invitation), and *Mary Josephine Waddell* (by invitation).
41. Anabolic Effects of Bovine Growth Hormone in Man.
Ivar Werker (by invitation), and *Frederic C. Bartter.*
42. Evidence for Stimulation of Insulin Secretion by Growth Hormone (STH) in the Rat.
Frank L. Engel, and *Thomas Albertson* (by invitation), *Joan Fredericks,* and *Enrique Lopez.*

INTERMISSION

43. Differential Release of ACTH *in vivo* by a Hypophysiotropic Fraction and Purified Lysine Vasopressin.
Roger Guillemin, G. W. Clayton, J. D. Smith (by invitation), *H. S. Lipscomb* (by invitation), *D. N. Ward* (by invitation), *A. V. Schally* (by invitation).
44. Mechanism of Inhibition of ACTH Release by Hydrocortisone.
A. Brodish and *C. N. H. Long.*
45. Effects of ACTH upon Pituitary Responsiveness to Stress after Steroid Therapy in Rats.
Julian I. Kitay, Donald A. Holub (by invitation), and *Joseph W. Jailer.*

46. Some Effects of Hormones on Electrical Activity and Thresholds in the Brain.
M. Kawakami (by invitation), and *Charles H. Sawyer.*

47. Precocious Estrus in Rats with Hypothalamic Lesions.
E. M. Bogdanove, and *H. C. Schoen* (by invitation).

48. Gonadotrophic Activity of the Periodic Acid-Schiff Reactive Material of Anterior Pituitary "Basophils" of Normal Rats.
Griff T. Ross (by invitation), *Robert C. Bahn* and *Robert W. Schmidt* (by invitation).

FRIDAY, JUNE 20, 1958

1:30 P.M.—ANNUAL BUSINESS MEETING

V. 2:30 P.M.—GONADS AND REPRODUCTION

Italian Room, St. Francis Hotel

Chairmen: *Ralph I. Dorfman and Herbert Kupperman*

49. Urinary Gonadotropin Excretion Levels Measured by the Mouse Uterine Response Employing Estrone as the Standard Reference Material.
Eugenia Rosemberg.

50. A New Assay for Gonadotropins.
Warner H. Florsheim, Shirley M. Velcoff (by invitation), and *Ralph E. Bodfish* (by invitation).

51. Luteinizing Hormone and Ovulation.
Sheldon J. Segal.

52. Follicle Stimulation and Luteinization in Hypophysectomized Rhesus Monkeys.
E. Knobil, J. L. Kostyo (by invitation) and *R. O. Greep.*

53. Effect of Folic Acid and Glutamine Antagonists on Chorionic Gonadotropin Producing Tumors.
C. H. Li, and *G. B. Magill* (by invitation).

54. Effect of Triethylenemelamine on Reproduction.
Emil Steinberger (by invitation), *W. O. Nelson,* and *A. Boccabella* (by invitation).

INTERMISSION

55. Steroidal Hydroxylases of the Human Adrenal Gland.
Alfred M. Bongiovanni, and *Walter R. Eberlein.*

56. α-Ketolic Estrogen (16α-Hydroxyesterone) Titers in Human Pregnancy Urine.
Benjamin F. Stimmel.

57. Steroid Ring A Aromatization by Human Placental Microsomes.
Kenneth J. Ryan (introduced by Lewis L. Engel).

58. The Effect of FSH on the Conversion of Testosterone-C-14 to Estradiol-C-14 by Dog Ovarian Slides.
Nina Hollander (by invitation), and *Vincent P. Hollander.*

59. Pharmacologic and Clinical Appraisal of the Newer Progestational Steroids.
Jeanne A. Epstein, Jean Seidl (by invitation), *Arthur Cutler* (by invitation) and *Herbert S. Kupperman.*

60. Masculinization of the Female Fetus Associated with Administration of Oral and Intramuscular Progestins During Gestation.
Lawson Wilkins, Howard W. Jones, Jr. (by invitation), *Gerald H. Holman* (by invitation), and *Robert S. Stempfel, Jr.* (by invitation).

VI. 2:30 P.M.—DIABETES AND METABOLISM

Colonial Room, St. Francis Hotel

Chairmen: *F. D. W. Lukens and Frank Engel*

THOSE REGISTERED FOR THE MEETING OF THE AMERICAN DIABETES ASSOCIATION WILL BE ADMITTED TO THIS MEETING

61. The Effect of Insulin on Serum Lipoprotein Concentrations in Diabetic Patients.
Edward Strisower, (introduced by F. D. W. Lukens).
62. Comparison of the Effects of Starvation and Alloxan Diabetes on Palmitic Acid Oxidation by Muscle.
Irving B. Fritz, and *Harold Dundee*, (by invitation).
63. The Effect of Various Steroids of the Hyperglycemic Action of Glucagon.
Shirley Weisenfeld, and *Martin G. Goldner*.
64. Mechanism of the Glycosuria Produced by Repeated Injections of Glucagon in Normal Subjects.
Jean-Pierre Felber and *Theodore B. Van Itallie* (introduced by F. D. W. Lukens).
65. Interrelationship Between Glucagon and Compound F in Protein Metabolism of Adrenalectomized Fasting Rats.
Stanley R. Glasser, and *Joseph H. Izzo* (by invitation).
66. The Effect of Insulin on Hepatic Glucose Production in Normal Dogs.
Robert Mahler, *William C. Shoemaker*, and *Daniel E. Pughs* (introduced by James Ashmore).

INTERMISSION

67. Turnover and Oxidation of Plasma Glucose in Humans as Influenced by Insulin and Orinase.
G. L. Searle, *G. E. Mortimore*, *R. Buckley*, and *W. A. Reilly* (introduced by F. D. W. Lukens).
68. The Effect of 6-Deoxy-6-Fluoroglucose on the Metabolism of Glucose.
Arne N. Wick (by invitation), *George S. Serif* (by invitation), *Ellen Larson* (by invitation) and *Charles J. Stewart* (by invitation).
69. Effects of 2-Deoxyglucose on Glucose Metabolism in the Rat and on the Hypoglycemic Response to Tolbutamide (Orinase).
Josiah Brown, and *Howard L. Bachrach* (by invitation).
70. Pattern of Carbohydrate and Fat Metabolism During Prolonged, Effective Doses of Human Growth Hormone in Man.
Dorothy H. Nenneman (introduced by Maurice Pechet).
71. The Inhibition of Insulin Degradation by Plasma Fractions of Non-Diabetic Sera.
Charles J. Goodner (by invitation), *Sidney H. Ingbar*, and *Norbert Freinkel*.
72. Endogenous Insulin Secretory Response to Hyperglycemia as Evidenced by Acute Changes in Plasma Glucose-C^{14}-Concentration.
S. A. Berson, *S. Weisenfeld*, and *M. Pascullo* (by invitation).

VII. ANNUAL DINNER—St. Francis Hotel

Leo Samuels, President, presiding

6:30 P.M.—Cocktails. Sponsored by the donors of the Awards of the Society: Ciba, Schering, Ayerst, Upjohn and Squibb. Attandance limited to holders of dinner tickets.

7:30 P.M.—Dinner

Presentation of 1958 Awards of the Society by E. B. Astwood:
The Endocrine Medal
Ciba Award
Squibb Fellowship
Schering Scholar
Upjohn Scholar
President's Address: Dr. Samuels

SATURDAY, JUNE 21, 1958

8:30 A.M.—Registration—Mezzanine, St. Francis Hotel

9:00 A.M.—OPENING SCIENTIFIC SESSION OF THE AMERICAN DIABETES ASSOCIATION WILL BE HELD AT THE MARK HOPKINS HOTEL. ENDOCRINE SOCIETY BADGE WILL ADMIT YOU TO THIS MEETING.

VIII. 9:00 A.M.—ADRENAL

Colonial Room, St. Francis Hotel

Chairmen: *John A. Luetscher, Jr., and Alfred M. Bongiovanni*

73. Adrenal Ascorbic Acid Depletion and Corticosteroid Release in Hypophysectomized Rats with Low Doses of ACTH.
Margaret A. Slusher (by invitation) and *Sidney Roberts.*
74. Potentiation of ACTH by Corticosteroid Precursors.
Irene T. Kline and *Richmond W. Smith, Jr.*
75. Pituitary ACTH and Plasma Free Corticosteroids During Regeneration of the Enucleated Rat Adrenal Gland.
Claude Fortier and *Jack De Groot* (by invitation).
76. Studies on the Functional Analysis of Hyperactive Adrenal States.
Vincent C. Di Raimondo, Dace B. Mitchell (by invitation), *Shirley Cook* (by invitation) and *Peter H. Forsham.*
77. Effect of Various Pituitary and Hypothalamic Extracts on Aldosterone Secretion.
E. H. Venning, O. J. Lucis (by invitation), *I. Dyrenfurth* (by invitation), and *J. C. Beck.*
78. Effects of Stress, Corticotropin, and Steroids of the Adrenal Necrosis of Besnoitia-Infected Hamsters.
J. K. Frenkel (introduced by F. R. Skelton).

INTERMISSION

79. Chronic Adrenocortical Dysfunction, Including Aldosterone Deficiency: Studies of Steroid and Electrolyte Metabolism.
George D. Molnar (by invitation), *Vernon P. Mattox* (by invitation), *Harold L. Mason,* and *Marschelle H. Power* (by invitation).
80. Aldosterone Excretion in Patients with Virilizing Adrenal Hyperplasia with Normal and Low Salt Intake.
Robert M. Blizzard (by invitation), *Grant W. Liddle,* and *Claude J. Migeon.*
81. Aldosterone Secretion in Dogs with Hypothalamic Lesions.
W. F. Ganong, A. H. Liberman (by invitation), *P. J. Mulrow* (by invitation), and *J. A. Luetscher, Jr.*

82. The Metabolism of Tritiated Aldosterone in Man.
Bernard Kliman, and *Ralph E. Peterson* (introduced by G. Donald Whedon).
83. Chronic Aldosterone Administration in Normal Subjects.
J. Thomas August (by invitation), *Don H. Nelson*, and *George W. Thorn*.
84. Primary Hyperaldosteronism Due to Hyperfunction of the Adrenal Cortex Corrected by Partial Adrenalectomy.
B. J. Kennedy, James Melby, Frederick Goetz, Walter Moran (by invitation), and *Bernard Zimmerman*.

SATURDAY, JUNE 21, 1958

CLINICAL PANELS

IX. 2:00 P.M.—ENDOCRINE TREATMENT OF NEOPLASTIC DISEASE

Colonial Room, St. Francis Hotel

Chairman: *O. H. Pearson*

William H. Baker — *Herbert Brendler*
Albert Segaloff — *B. J. Kennedy*
Delbert Bergenstal

X. 2:00—HYPERPARATHYROIDISM AND BONE DISEASE

Italian Room, St. Francis Hotel

Chairman: *Gilbert S. Gordon*

John Eager Howard — *Philip Henneman*
Laurence H. Kyle — *Oliver Cope*
W. P. Laird Myers

XI. 3:30—CHEMICAL TREATMENT OF DIABETES

Colonial Room

THOSE REGISTERED FOR THE MEETING OF THE AMERICAN DIABETES ASSOCIATION WILL BE ADMITTED TO THIS MEETING

Chairman: *Peter Forsham*

Alexander Marble — *Albert E. Renold*
Max Miller — *Henry Dolger*
Robert Williams

XII. 3:30—INTERSEXUALITY

Italian Room, St. Francis Hotel

Chairman: *Warren Nelson*

Melvin Grumbach — *Herbert Kupperman*
R. Hoffenberg — *Robert Greenblatt*

TO BE READ BY TITLE

* See Abstracts 85–188

* Abstracts of Papers appearing on Program and those read by title will not be printed in the *Journals* this year but will be printed in full in the Annual Program.

THIRD WORLD CONGRESS ON FERTILITY AND STERILITY

The Third World Congress on Fertility and Sterility, sponsored by the International Fertility Association, will be held in Amsterdam, Holland, from June 7 to 13, 1959.

The general outlines of the sections of the program will be as follows:

1. Female sterility (Physiology of reproduction, pathology, endocrinology, clinical problems, treatment).
   clinical problems, treatment).
2. Male sterility (Physiology of reproduction, pathology, endocrinology, clinical problems, treatment).
3. Basic research and/or animal reproduction.
4. Psycho-sexual problems.

Although any original report on some phase of fertility and infertility, either clinical or in the field of the basic sciences, will be considered, definite priority will be given to those papers concerning the following subjects:

1. Embryonic death (Etiology, pathogenesis, placental structures in its relation to the condition of the foetus, functional problems and diagnosis of the embryonic and foetal death, and habitual abortion).

2. Hormonal factors and vitamins in fertility and sterility (Ovulation and sterility, induction of ovulation, influence of thyroid, steroids etc. on ovulation, influence and physic-pathological significance of vitamins, hormones and spermatogenesis, vitamins and spermatogenesis).

3. Relative value of the techniques for study of the endocrine functions in human sterility (Study of the estrogen function, study of the luteal function, study of the adeno-hypophysis function, testicular biopsy value, study "in vitro" of the fertilization of mammalian and human ovums, use of radio-isotope designers in the study of the sexual function).

4. Biochemistry of spermatogenesis.

5. Psycho-sexual problems in sterility.

Official languages: English, French, German, Spanish (with simultaneous translation).

Registration fee: full member U. S. $40.—
accompanying family-members: U. S. $15.—
after January 1st, 1959, the registration fee for full members will be U. S. $50.—.

Titles for papers should be sent not later than June, 1958, to both Dr. Alfonso Alvarez-Bravo, Avenida Horacio 1506, Mexico City 5, D.F.,

Mexico, chairman, and Prof. Dr. B. S. ten Berge, Academisch Ziekenhuis, Groningen, Holland, executive member of the Program Committee.

For further information and registration apply to Dr. L. I. Swaab, Hon. Secretary Third World Congress on Fertility and Sterility, Sint Agnietenstraat 4, Amsterdam-C. or to the nearest Wagons Lits/Cook office.

THE GERONTOLOGICAL SOCIETY, INC. NOTICE OF MEETING

The eleventh annual scientific meeting of the Gerontological Society, Inc., will be held at the Bellevue Stratford Hotel, Philadelphia, Pennsylvania, November 6, 7, and 8, 1958.

Abstracts of papers for the program should be submitted to the Program Committee for consideration by July 1, 1958. Abstracts should be sent to the sub-chairmen of the section in which the author(s) elect to give their paper.

The sub-chairmen are: *Clinical Medicine*—Dr. Ewald Busse, Duke University Hospital, Durham, North Carolina; *Biology*—Dr. Morris Rockstein, Department of Physiology, New York University, 550 First Avenue, New York 16, New York; *Psychology*—Dr. Ethel Shanas, National Opinion Research Center, 5711 South Woodlawn Avenue, Chicago, Illinois; and *Sociology*—Dr. W. M. Beattie, Jr., Department of Sociology, Washington University, St. Louis, Missouri.

INTER-SOCIETY CYTOLOGY COUNCIL

Inter-Society Cytology Council, Annual Scientific Meeting, Hotel Statler, New York, New York, November 13, 14, 15, 1958. Paul F. Fletcher, M.D., Secretary, 634 North Grand Avenue, St. Louis 3, Missouri.